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Publications list

Articles

Bassa,C., Mila,I., Bouzayen,M. and Audran-Delalande,C., (2012) Phenotypes associated with down-regulation of *Sl-IAA27* support functional diversity among Aux/IAA family members in the tomato. *Plant Cell Physiol.* 53(9): 1583-1595. Cover of September issue.

Audran-Delalande, C., **Bassa, C.**, Mila, I., Regad, F., Zouine, M. and Bouzayen, M. (2012) Genome-Wide Identification, Functional Analysis and Expression Profiling of the Aux/IAA Gene Family in Tomato. *Plant Cell Physiol*. 53: 659–672.

Posters

Bassa,C., Delalande,C., Van Der Rest,B., Wang,X., Buscaille,P., and Bouzayen,M., (2011) Sl-IAA27 protein is involved in tomato root and fruit development and in leaf chlorophyll content. Jacques Monod Conference "Evolution of plant developmental regulatory mechanisms". Roscoff, France.

Bassa,C., Delalande,C., Van Der Rest,B. and Bouzayen,M., (2011) Auxin signaling function during tomato development: functional caracterisation of *Sl-IAA27*. Conference of SEVAB PhD school. Toulouse, France.

Bassa,C., Delalande,C. and Bouzayen,M., (2011) Silencing of *Sl-IAA27* results in modified chlorophyll content in tomato leaves. Journées inter-fédérations en sciences du vivant. Toulouse, France.

Oral communications

Bassa,C., Delalande,C., Van Der Rest,B., Wang,X., Buscaill,P. and Bouzayen,M., (2012) Auxin regulation of tomato fruit development: function of the *Sl-IAA27* gene. Journée de la recherche ENSAT (Ecole Nationale Supérieure Agronomique de Toulouse). Toulouse, France.

Delalande,C., **Bassa C.,** Mila,I., Li,Z., Van der Rest,B. and Bouzayen,M., (2010). The auxin control of fruit development in tomato is mediated by the Aux/IAA transcriptional regulators. Indo French Symposium, Genomics and biotechnology of fruit quality: recent advances, Lucknow, India.

Résumé

Au cours du développement des plantes, l'auxine contrôle de nombreux processus dont notamment la dominance apicale, le phototropisme, la phyllotaxie, la formation des racines latérales et le développement des fruits. Le métabolisme, la perception ainsi que la signalisation de l'auxine ont majoritairement été étudiés chez Arabidopsis. Afin d'élucider la fonction de cette hormone au cours du développement des fruits, nous avons utilisé la tomate comme plante modèle. En effet la tomate est à la fois une espèce référence pour la famille des Solanacées mais également pour les plantes à fruits charnus. Les gènes *Aux/IAA* jouent un rôle déterminant dans la voie de signalisation auxinique en formant notamment un complexe avec l'un des récepteurs de cette hormone et en réprimant l'activité des facteurs de transcriptions de type ARF. Au cours de ce travail, nous avons caractérisé la famille multigènique des *Aux/IAA* chez la tomate. Elle est composée de 25 membres que nous avons nommés en référence à ceux d'Arabidopsis. Le niveau d'expression des gènes *Aux/IAA* est variable en fonction du gène, de l'organe ou du tissu considéré. L'expression de plusieurs de ces gènes est régulée à la fois par l'auxine et l'éthylène, ce qui suggère que les *Aux/IAA* sont reliés aux voies de signalisation de ces deux hormones. L'élucidation de la fonction des Aux/IAA de tomate est réalisée à travers la caractérisation de plantes transgéniques avec une attention particulière portée aux lignées montrant des phénotypes affectant le développement et la qualité du fruit ou présentant un intérêt pour le dialogue entre l'auxine et l'éthylène. Parmi ces lignées, les plantes sous-exprimant le gène *Sl-IAA27* présentent une altération du développement des fleurs et des fruits. De plus, la diminution de l'expression de *Sl-IAA27* entraine la sous-expression de gènes impliqués dans la voie de biosynthèse de la chlorophylle se traduisant par une diminution de la teneur en chlorophylle dans les feuilles. Ces résultats montrent la diversité fonctionnelle des Sl-IAA et souligne le rôle de régulateur joué par l'auxine au cours du développement du fruit.

Abstract

The phytohormone auxin controls various developmental processes, including apical dominance, tropisms, vascular patterning and fruit set. Auxin metabolism, transport, perception and signaling are mainly studied in the plant model Arabidopsis. To understand the auxin regulation process of fruit development, the tomato plant which is a reference species for Solanaceae and fleshy fruit plants is a good model of study. *Aux/IAA* genes play a key role in auxin signaling pathway, through their participation to the receptor complex of the hormone and by repressing the activity of A*RF* type transcription factors. In this work the 25 *Sl-IAA* family members have been isolated and renamed according to their phylogeny relationship with *AtIAAs*. *Sl-IAA* genes display distinctive expression pattern in different tomato organs and tissues, and some of them display differential responses to auxin and ethylene, suggesting that *Aux/IAAs* may play a role in linking both hormone signaling pathways. To improve knowledge about *Aux/IAA* function, transgenic tomato plants have been generated. The involvement of *Aux/IAAs* in notably fruit development was addressed through the characterization of the *Sl-IAA27* gene. Its down-regulation in plants lead to altered flower and fruit development with a modified shape of the fruits and reduced volume. Moreover, fertilization capacity was strongly altered by the silencing of *Sl-IAA27* resulting in the formation of fruits with reduced seed number. In addition, the reduced expression of *Sl-IAA27* leads to a down-regulation of genes involved in chlorophyll biosynthesis triggering reduced leaf chlorophyll accumulation content. These results showed a functional diversity among *Sl-IAA* family members and underlined the involvement of auxin notably in the regulation of fruit development.

Sommaire

Abreviations

2.4 D: 2.4-dichlorophenoxyacetic acid

4-Cl-IAA: 4-Chloroindole-3-acetic acid

AAO: ACETALDEHYDE OXIDASES

ABA: Abscisic Acid

ABCB/PGP: ATP-binding cassette transporters/ multi-drug resistance/P-glycoprotein

ABP1: AUXIN BINDING PROTEIN 1

ACC: 1-aminocyclopropane-1-carboxylic acid

AFB: Auxin Receptor F-box

Ala: Alanine

AMI1: AMIDASE 1

ARF: Auxin Response Factor

ARR: type A Arabidopsis Response Regulator

Asp: Aspartate

Aux/IAA : Auxin/Indole-3-Acetic Acid

AUX/LAX : AUXIN RESISTANT 1/LIKE AUX1

AuxRE: Auxin Responsive cis-Element

BFA: Fungal toxin brefeldin A

BR: Brassinosteroid

CaMV: *Cauliflower Mosaic Virus*

CK: Cytokinin

CPI1: Cyclopropylsterol Isomerase1

CYP: Cytochrome P450

DOR: DROUGHT TOLERANCE REPRESSOR

DPB: Dimerization Protein B

E2FC: E2 promoter binding factor C

EAR: Ethylene-responsive element binding factor-associated Amphiphilic Repression

ER: Endoplasmic Reticulum

EST: Expression Sequence Tag

GA: Gibberellin

GH3: Gretchen Hagen 3

Glu: Glutamate

GNL1: GNOMLIKE1 regulation

GFP: Green Fluorescent Protein

IAA: Indole-3-Acetic Acid

Iaglu: IAA glucose synthase

IAA-glucose: 1-O-indol-3-ylacetyl-beta-D-glucose

IAAId: Indole-3-acetaldehyde

IaaM: TRYPTOPHAN-2-MOOXYGENASE

IAM: Indole-3-acetamide

IAA-myo-inositol: Indole-3-ylacetyl-myo-inositol

IAN: Indole-3-acetonitrile

IAOx: Indole-3-acetaldoxime

IBA: Indole-3-butyric acid

IPA: Indole-3-propionic acid

IPA: Indole-3-pyruvate

JA: Jasmonate

LEC: LEAFY COTYLEDON

Leu: Leucine

LR: Lateral Root

MS medium: Murashige and Skoog medium

NAA: Naphthalene-1-Acetic Acid

NGA: NGATGAs

NIT: Nitrilase

NLS: Nuclear Localization Signal

NPA: N-1-napthylphtalamic acid

Ox-IAA-Asp: Oxindole-3-acetyl-NAsp

OH-IAA-Asp: Oxindole-3-acetic acid

PBA: Pyrenoyl Benzoic Acid

PDK1: 3-Phosphoinositide-Dependent Protein Kinase 1

Phe: Phenylalanine

PHOT1: PHOTOTROPIN1

PIF4: Phytochrome-Interacting Factor 4

PIN: PINFORMED

PILS: PIN-LIKEs

PLP-dependent: Pyridoxal-phosphate-dependent

PM: Plasma Membrane

PP2A: Protein Phosphatase 2A

qRT–PCR : quantitative Reverse Transcription–PCR

RBX1: RING BOX1

RNAi: RNA interference

RMS: RAMOSUS

SAM: S-Adenosyl- L-Methionine

SAUR: Small Auxin Up RNA

SCF: SKP1-Cullin-F-box

SGN: Solanaceae Genomics Network

SHI: SHORT INTENOTES

SKP1: SUPPRESSOR OF KINETOCHORE PROTEIN 1

SKP2A: S-PHASE KINASE-ASSOCIATED PROTEIN 2A

Sl-IAA: *Solanum lycopersicum* Auxin/Indole-3-Acetic Acid

SMT1: STEROL METHYLTRANSFERASE1

SNX1: SORTING NEXIN1

SPL: SPOROCYTELESS

STY1: STYLISH1

TAAR: TIR1 and AFB1-2-3 paralogs

TAM: Tryptamine

TDC: TRP DECARBOXYLASE

TIBA: Triiodobenzoic Acid

TIR1: Transport Inhibitor Resistant1

TPL: Topless

Trp: Tryptophane

YFP: Yellow Fluorescent Protein

General introduction of the thesis

The phytohorme auxin is a key regulator of many plant development processes, including cell division, elongation, rhyzogenesis, apical dominance and organ patterning but its action of fruit development remains poorly elucidated. To improve our knowledge about how auxin is able to regulate fruit development it is necessary to understand specific function of molecular components involved in auxin signaling. Among theses the *Aux/IAA* genes family plays a pivotal role by notably repressing the activity of the auxin responsive genes regulators, the A*RF* transcription factors. To date most of our understanding about *Aux/IAAs* physiological significance comes from the analysis of gain-of-function Arabidopsis mutants. Nevertheless Arabidopsis is not a suitable model to analyze fruit development and due to numerous resources available the tomato appears to be a good complementary model. The purpose of my PhD was first to identify all *Aux/IAA* genes in tomato thanks notably to data available from the tomato genome sequencing and to analyse their pattern of expression. The second purpose was to determine the role of *Aux/IAAs* in fruit development and in the cross-talk regulation by auxin and ethylene. After phenotypic analyses performed on four different *Aux/IAAs* under-expressing tomato transgenic lines, my work was focused on the *Sl-IAA27* gene which appeared to be a key regulator of fruit development.

The first chapter consists in a bibliographic review on auxin. It is presented in this chapter actual knowledge regarding auxin metabolism, transport, perception and signaling. It describes main roles of auxin on the regulation of different plant development processes and its interaction with other phytohormones to mediate it. In this part, it is also explained why the tomato plant was choosen as plant model to study auxin action on fruit development. More

specifically the characteristics of the *MicroTom* cultivar mainly used for analysis realized are described in this part. The following chapters focus on the characterization of the *Aux/IAA* genes family in tomato, these genes being one of the major players of auxin signaling. The chapter II is mainly composed by an article published in the Plant and Cell Physiology journal presenting the 25 genes members of *Sl-IAA* family from which seven members were identified during this PhD thanks to the tomato genome sequencing. Phylogenetic analyses between Sl-IAAs and At-IAAs allowed the determining of Sl-IAA names and clades division. Aux/IAA family is less important in tomato than in Arabidopsis with 25 and 29 members respectively due notably to reduced non canonical Aux/IAA number in tomato. Analysis of *Sl-IAAs* chromosomal distribution revealed that *Sl-IAAs* duplication may have occurred during evolution in tomato. The study of tomato pattern of expression for most of *Sl-IAAs* showed that only eight of them are expressed during fruit development. Moreover, no link was found between phylogenetic relationships among *Sl-IAAs* and their pattern of expression. Transient expression in single cell system (tobacco protoplasts) showed that all Sl-IAAs present a repressor activity but not with same strength for each. No correlation was found between the level of Aux/IAA repressor activity and domain I structure. In addition nuclear targeting was observed for three of Sl-IAA tested but it was also detected in cytoplasm in the case of the non canonical Sl-IAA32 protein. This data indicated that at least one of Sl-IAAs could have extranuclear function which nevertheless remains to be explored. The determining of the regulation of *Sl-IAAs* expression upon either auxin or ethylene treatments revealed that most of these genes could be regulated by both hormones. This is in support with the idea that *Aux/IAAs* could mediate auxin and ethylene cross-talk notably during fruit development. To elucidate physiological significance of Aux/IAA during fruit development a reverse genetic approach was chosen. The chapter III describes all the transgenic plants generated. Three *Sl-*

IAAs (*Sl-IAA3*, *Sl-IAA9* and *Sl-IAA15)* were previously characterized and their down-regulation showed specific phenotypes. Phenotypic analyses showed notably significative altered fruit development of *Sl-IAA27* RNAi plants. Most of the chapter III is then presented on the form of an article which has been accepted for publication in the Plant and Cell Physiology journal. This paper presents most of the results obtained regarding *Sl-IAA27* phenotypic and molecular characterization. It is notably shown that the normal *Sl-IAA27* expression is required for proper fertilization process and for normal fruit development. In addition this gene seems able to regulate chlorophyll biosynthesis in leaves showing that auxin may also control this process. Contrary to most of *Aux/IAA* genes, the expression of *Sl-IAA27* is repressed upon auxin accumulation and its promoter is not auxin-inducible. Among all Sl-IAA members, Sl-IAA27 display most closed structure with Sl-IAA9 which has previously shown as a key regulator of fruit initiation. Analysis of Sl-IAA27 and Sl-IAA9 protein structure revealed common conserved domain YxGLS at the N amino terminal part, before domain I, which is conserved in their homologues in all species studied, suggesting that the presence of this domain may be related to their function as regulator of fruit development.

Chapter I: Bibliographic review

Résumé du chapitre I

Le premier chapitre de cette thèse consiste en une revue bibliographique décrivant les avancées scientifiques actuelles concernant l'auxine. Ce chapitre est divisé en six parties décrivant respectivement le métabolisme de l'auxine, son transport au sein de la plante, ses différents récepteurs et voie de signalisation, les différents rôles de l'auxine, ses interactions avec les autres hormones ainsi que les caractéristiques et intérêt de la tomate. L'auxine peut être synthétisée soit par une voie indépendante du tryptophane qui est peu décrite à ce jour soit par quatre voies parallèles utilisant le tryptophane comme précurseur. L'auxine est la seule hormone décrite à ce jour qui est en partie transportée de manière active au sein de la plante. Ce transport polaire fait intervenir quatre familles de transporteurs, ceux de type AUX1/LAX permettant l'entrée de l'auxine dans la cellule, les PINs, PILS et ABCBs prenant en charge la sortie de l'auxine en dehors de la cellule ainsi que le contrôle de l'homéostasie intercellulaire. L'auxine peut être perçue par différent récepteurs, ABP1, SKP2A et TIR1. La voie de signalisation en aval de la perception de l'auxine par ABP1 reste hypothétique jusqu'à présent. La perception par TIR1 de l'auxine est traduite au niveau d'une voie de signalisation faisant intervenir deux familles multigéniques de facteurs de transcription, les *ARFs* et les *Aux/IAAs* ainsi que des co-répresseurs de type *TOPLESS*. L'auxine régule de nombreux processus de développement dans la plante dont notamment la division et élongation cellulaire, l'architecture racinaire, la mise en place des organes végétatifs et floraux ainsi que la pollinisation et mise à fruit. Afin de contrôler ces différents mécanismes, l'auxine interagit avec d'autres phytohormones de manière tissu spécifique. La tomate, en tant qu'espèce référence pour les Solanacées et les plantes à fruits charnues, constitue un bon modèle d'étude pour analyser la régulation auxinique du développement du fruit. Enfin le cultivar MicroTom

principalement utilisé pour les études réalisées lors de cette thèse a un phénotype nain, un cycle cellulaire plus rapide et est facilement transformable via *Agrobacterium*.

First observations of auxin regulation of plant development were made by Charles Darwin who observed that a plant substance was able to modulate plant shoot elongation to allow tropic growth toward light (Darwin 1880). This substance was then isolated and called auxin, its major form being indole-3-acetic acid (IAA) (Thimann 1977). Numerous studies have then improved the knowledge about how this phytohormone is synthesized, transported and able to regulate so many plant development processes.

I Auxin metabolism

Several molecules display auxin activity such as indole-3-butyric acid (IBA), 4-Chloroindole-3-acetic acid (4-Cl-IAA) and indole-3-propionic acid (IPA). Nevertheless, the major auxin form found in plants is indole-3-acetic acid (IAA).

I.1 Synthesis pathway

Analysis of auxin overproduction mutants in Arabidopsis revealed different pathways of auxin biosynthesis involving a key amino acid as precursor, the tryptophane (Trp) (Bartel 1997). Therefore Trp mutants are mainly used to study auxin biosynthesis. However, there is no difference in free IAA levels between wild type and Trp mutants showing that IAA could also be synthesized by a Trp independent pathway. Recent studies on Trp auxotroph mutants showed that Trp dependent and independent pathways both contribute to IAA biosynthesis in shoots but that Trp dependent pathway is predominant in roots and is responsible for root gravitropism (Kiyohara et al. 2011).

I.1.1 Tryptophane independent pathway

Despite we know that Trp independent synthesis of auxin occurs in specific tissues knowledge about genes involved in this pathway remains poor until now. To date, the subcellular

localization of this auxin synthesis pathway is not clear while some analyses suggest that it should occur in plastid (Rapparini et al. 2002). Analysis of *sulfurea* mutant revealed that *sulfurea* gene should be implied in Trp independent auxin synthesis pathway. Indeed, this mutant displays auxotrophy phenotypes and altered auxin level while auxin Trp dependent pathway is not altered. Moreover auxin transport and signaling remain unmodified in *sulfurea* mutant. Nevertheless the involvement of *sulfurea* gene as an enzyme used in auxin Trp independent synthesis or as a regulator of this pathway is not resolved yet (Ehlert et al. 2008).

I.1.2 Tryptophane dependent pathways

The trp dependent IAA biosynthesis can be divided in four different parallel pathways (Figure 1).

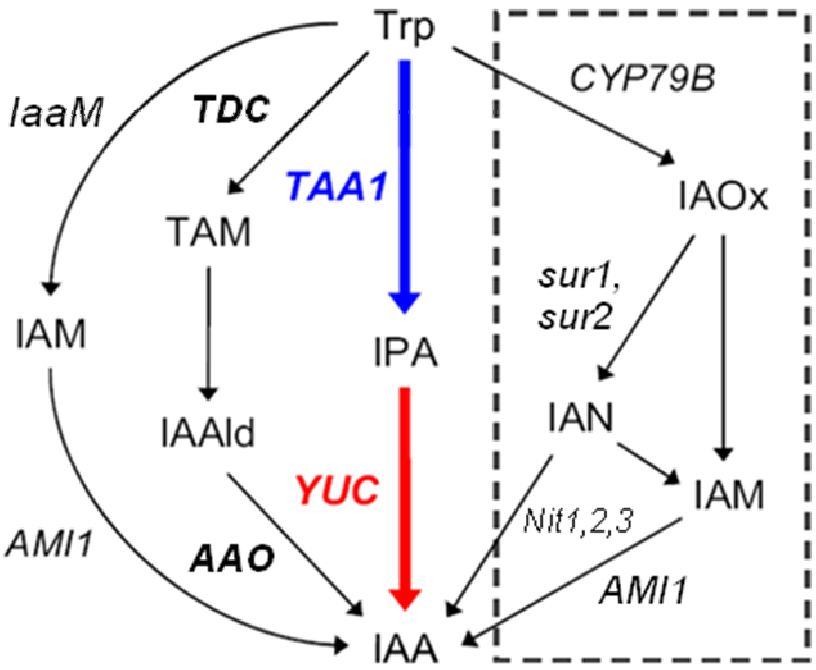

Figure 1: Trytophane dependent synthesis pathway (Mashiguchi et al. 2011 modified)

I.1.2.1 IAA synthesis using indole-3-acetaldoxime (IAOx) as an intermediate

In this pathway Trp is directly converted into IAOx which can be then used to make indole-3-acetonitrile (IAN) and indole-3-acetamide (IAM) (Figure 1). The IAOx pathway was identified by the characterization of three auxin overproduction mutants, *superoot1 (sur1)* (Boerjan et al. 1995), *superoot2 (sur2 or CYP83B1)* (Delarue et al. 1998; Barlier et al. 2000; Morant et al. 2010) and *Cytochrome P450 79B2* (*CYP79B2)* (Hull et al. 2000; Zhao et al. 2002; Mikkelsen et al. 2009; Bak et al. 2011). The gene *CYP79B2* and its closed homolog *CYP79B3* allow the direct conversion of Trp to IAOx (Sugawara et al. 2009) (Figure 1). *Sur1* and s*ur2* mutants display similar auxin overproduction phenotypes including formation of numerous adventitious roots from hypocotyls (Boerjan et al. 1995; Delarue et al. 1998) (Figure 2). These genes are both involved in IAOx conversion to IAN. IAN can then be converted in IAA by nitrilases (NIT1, NIT2, NIT3) (Normanly et al. 1997; Hillebrand et al. 1998; Vorwerk et al. 2001) (Figure 1).

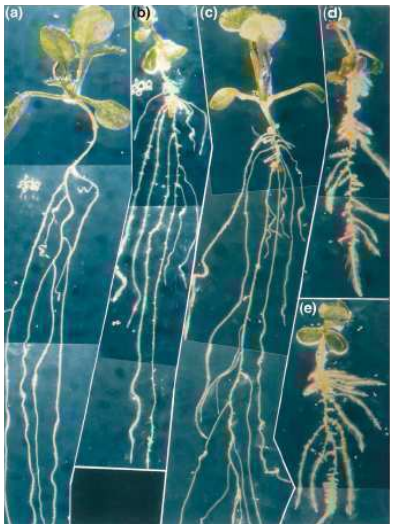

Figure 2: Phenotype of three week old plants of (a) wild-type, (b) *sur1* mutant, (c) *sur2* mutant, (d) (e) *sur1sur2* double mutant (Delarue et al., 1998).

The IAOx pathway seems mostly used in glucosinolate-producing plants as Arabidopsis, since it was shown that this synthesis pathway does not occur significantly in pea, rice, maize and tobacco with notably the absence of *CYP79B2* and *CYP79B3* homologs (Quittenden et al. 2009; Sugawara et al. 2009)

I.1.2.2 IAA synthesis using indole-3-pyruvate pathway (IPA) pathway

IAA can also be synthesized from Trp through IPA pathway. Trp is converted in IPA thanks to a Trp aminotransferase called TAA1 which is a pyridoxal-phosphate-dependent (PLP-dependent) enzyme (Figure 1). TAA1 plays critical roles during embryogenesis, flower development, seedling growth, vascular patterning, lateral root formation, tropism, shade avoidance, and temperature-dependent hypocotyl elongation (Stepanova et al. 2008; Won et al. 2011).

It was previously thought that *YUCCA* genes were involved in the conversion of tryptamine (TAM) to indole-3-acetaldehyde (IAAId). Nevertheless, it had been recently shown that *YUCCA* genes family is implicated in the conversion of IPA to IAA (Mashiguchi et al. 2011; Stepanova et al. 2011; Won et al. 2011) and that TAM and IAAId are involved in a distinct pathway (Figure 1). The *YUC1* gene was the first *YUCCA* gene identified, it was isolated from a screening of long hypocotyls Arabidopsis mutants defective in light signaling. The *YUC1* mutant displays auxin overproduction associated phenotypes including formation of massive roots. Later studies identified the 11 members of the *YUCCA* family in Arabidopsis. The over-expression of each of them leads to dramatic phenotypes showing that these genes are essential for seedling growth, leaf and flower initiation and vascular formation (Woodward et al. 2005; Cheng et al. 2006; Kim et al. 2007). Unlike *CYP79B2/B3* genes, *YUCCA* genes are

found in many plants notably in tomato (Expósito-Rodríguez et al. 2011), suggesting that IAA synthesis through IPA pathway is predominant in plant.

I.1.2.3 IAA synthesis using Tryptamine (TAM) pathway

The involvement of TAM pathway in auxin Trp dependent synthesis is known since long time but was until quite recently thought to be restricted to bacteria (Phelps and Sequeira 1967). TAM is produced from Trp thanks to TRP DECARBOXYLASE (TDC) (Lehmann et al. 2010). TAM is then directly converts to IAAId by unknown enzyme (Quittenden et al. 2009). The oxidation of IAAId to IAA is catalyzed by ACETALDEHYDE OXIDASES (AAOs) in bacteria (Figure 1). In Arabidopsis four *AAO* genes have been identified (Sekimoto et al. 1998; Akaba et al. 1999). Nevertheless the effective function of Arabidopsis AAO in auxin synthesis TAM pathway remains to be investigated, *AAO1* Arabidopsis mutant displaying no auxin associated phenotypes and no reduction in IAA levels (Seo et al. 2004).

I.1.2.4 Indole-3-acetamide (IAM) pathway

IAM can be a direct intermediate between Trp and auxin but could be also produced from IAOx. Nevertheless genes implied in converting IAOx to IAM are not known. The conversion of Trp to IAM is mediated by TRYPTOPHAN-2-MOOXYGENASE (IaaM) and is then converted to IAA by a IAM hydrolase, AMIDASE 1 (AMI1) (Spaepen et al. 2007) (Figure 1). The IAM pathway had been most characterized in bacteria and it was generally thought that this pathway was not used for IAA synthesis in plants. The identification of *AMI1* in Arabidopsis suggested that IAM could also be an intermediate for IAA synthesis in plants (Pollmann et al. 2003; Pollmann et al. 2006; Pollmann et al. 2009). Moreover, *AMI1* have also been identified in tobacco BY-2 cells and showed to be crucial for cell division,

demonstrating that because tobacco and Arabidospsis are not closely related plants IAM pathway is well used in plants kingdom (Nemoto et al. 2009; Mano et al. 2010).

Once synthesized only 25% of IAA remains in free form, major part being convert into IAA conjugates.

I.2 Production of auxin from auxin-conjugates

Auxin conjugates are divided in three groups, ester conjugates with sugar moieties, amide conjugates with amino acids and peptides (or proteins) conjugates. Plants are able to produce many different combinations of IAA conjugates (Bajguz and Piotrowska 2009).

Ester conjugates have been identified in many plant species. The synthesis of 1-O-indol-3-ylacetyl-beta-D-glucose (IAA-glucose) and indol-3-ylacetyl-myo-inositol (IAA-myo-inositol) have been first described in maize (Michalczuk and Bandurski 1982). Ester conjugates have been then detected in Arabidopsis (Tam et al. 2000; Jackson et al. 2001). The synthesis pathway of ester conjugates is described since long time and IAA glucose synthase (iaglu) was identified in maize and Arabidopsis (Michalczuk and Bandurski 1982; Szerszen et al. 1994; Jackson et al. 2001). IAA-myo-inositol is synthesized from IAA-glucose (Kowalczyk et al. 2003). The hydrolysis of ester conjugates remains undescribed until now.

Amide conjugates are detected in most plant species such as cucumber, soybean, Arabidopsis and *Physcomitrella patens*. The level and proportions between amide conjugates differ according to the plant species (Cohen 1982; Sonner and Purves 1985; Tam et al. 2000; Kowalczyk and Sandberg 2001; Ludwig-Müller et al. 2009). The synthesis of amide conjugates is mediated by the family of *Gretchen Hagen 3 (GH3)* and occurs in cytosol (Ludwig-Müller et al. 2009). In Arabidopsis *GH3* family is composed by 19 members from

which 7 have been shown to be able to catalyse the synthesis of IAA amide conjugates (Staswick et al. 2005). Each amide conjugates display a specific function. Auxin conjugates with aspartate (IAA-Asp) and glutamate (IAA-Glu) consist in a degradation intermediate of auxin. Indeed, when IAA level is high, IAA-Asp is oxidized to oxindole-3-acetyl-NAsp (Ox-IAA-Asp) and oxindole-3-acetic acid (OH-IAA-Asp) which are then degradated (Tuominen et al. 1994; Barratt et al. 1999). The conjugate between auxin and Trp (IAA-Trp) is not only a stored conjugate of auxin but plays a function of growth inhibitor. Indeed, IAA-Trp accumulation leads to agravitropic root growth in seedlings, and inhibits IAA effects on root growth and lateral root formation (Staswick 2009). Some amide conjugates such as with alanine (IAA-Ala), leucine (IAA-Leu) and phenylalanine (IAA-Phe) can be hydrolyzed back to free IAA (Kai et al. 2007).

Auxin can also be conjugated to peptides or proteins. This was first demonstrated in bean where IAA can bind the PvIAP1 protein (Bialek and Cohen 1986). Interestingly, this IAA-protein conjugation seems to be plant species specific thought that in Arabidopsis and *Medicago truncatula* IAA is not able to bound IAP1 homologs (Walz et al. 2008). Proteins which are able to be bound by IAA remain to be identified in Arabidopsis.

Auxin-conjugation is tissue-specific and mainly regulated. Indeed, genes involved in auxin conjugates synthesis and hydrolysis display tissue specific expression pattern and are regulated by abiotic and biotic stress and hormone treatments (Ludwig-Müller 2011).

I.3 Localisation of auxin synthesis

The localization of auxin synthesis plays a role in the regulation of auxin function. For example, local auxin biosynthesis is able to modulate gradient-directed polarity in root hair development in Arabidopsis (Ikeda et al. 2009). For long time it was thought that auxin

biosynthesis occurred only in shoots and that other tissues was dependent on auxin transport. Nevertheless both shoot and root can produce auxin (Cheng et al. 2006; Stepanova et al. 2008; Petersson et al. 2009). Indeed the *TAAI* gene which is implied in IPA pathway is expressed in both shoot and root (Stepanova et al. 2008). Moreover, in Arabidospsis, *YUCCA* genes are also expressed in all organs including flowers, leaves and roots. Regarding control of auxin gradient during development, each organ is self-sufficient. Indeed the effects of the inaction of *YUCCA2* and *YUCCA6* expression in stamens cannot be compensated by auxin production in other floral organs (Cheng et al. 2006). Moreover the expression of *YUCCA* and *TAA1* genes are restricted to a small group of cells. For example, in Arabidopsis, during embryogenesis at the globular stage they are expressed in the apical region to be gradually concentrated at the apical meristem at the heart stage and finally localized only in apical meristem in mature embryo (Cheng et al. 2006; Stepanova et al. 2008; Tao et al. 2008; Stepanova et al. 2011).

I.4 Regulation of auxin synthesis

Auxin synthesis could be regulated by both environmental and developmental signals. Indeed, when plants are moved from light to dark auxin synthesis is up-regulated (Tao et al. 2008). Other hormones can also act as regulator of auxin biosynthesis. For example, cytokinin was shown to positively regulate the expression of *TAA1* and *YUCCA6* genes involved in IPA pathway but also of *CYP79B2*, *CYP79B3* and *NIT3* implied in IAOx pathway (Jones et al. 2010; Zhou et al. 2011). At molecular level, genes involved in auxin biosynthesis pathways can be regulated by different transcription factors.

YUCCA genes can be regulated by *SHORT INTENOTES (SHI)* family of transcription factors. Over-expression of Arabidopsis *STYLISH1 (STY1)*, one member of *SHI* family, leads to

abnormal style development and vascular patterning and in increase of *YUCCA4* expression at transcriptional level (Sohlberg et al. 2006). *NGATGAs (NGA)* transcription factors are also able to regulate *YUCCA* genes. In Arabidopsis *NGA* quadruple mutant presents abolished formation of style and stigma tissues and displays low expression of *YUCCA2* and *YUCCA4* genes (Trigueros et al. 2009). *LEAFY COTYLEDON2 (LEC2)* which is a central regulator of embryogenesis is also able to activate expression of *YUCCA2* and *YUCCA4* genes and LEC2 can directly bound *YUCCA4* promoter (Stone et al. 2008). Moreover over-expression of *phytochrome-interacting factor 4 (PIF4)* results in increased expression of *YUCCA8* gene. Gel shift and chromatin immunoprecipitation showed that PIF4 can directly bind to a G-box in the promoter of *YUCCA8* gene (Sun et al. 2012). The over-expression of *SPOROCYTELESS (SPL*) leads to the repression of *YUCCA2* and *YUCCA6* genes expression suggesting that this gene is a negative regulator of *YUCCA* genes (Li et al. 2008). In conclusion, STY1, NGA, LEC2 and PIF4 can activate *YUCCA* genes expression while SPL is able to repress it.

The *LEAFY COTYLEDON 1 (LEC1)* transcription factor which, as *LEC2*, is a central regulator of embryogenesis negatively regulates *AMI1* expression (Verdier and Thompson 2008). Moreover AMI1 expression is induced in *AUXIN RESPONSE FACTOR (ARF) arf6arf8* double null mutants (Nagpal et al. 2005) and down-regulated in *CONTITUTIVE PHOTOMORPHOGNIC 9 (COP9) signalosome (CSN)* mutants (Dohmann et al. 2008).

II Auxin transport

Due to that auxin synthesis occurs in some specific tissues it has to be transported to be present in all the plant. While most plant hormones move passively in plants auxin is also actively transported in a polar way from aerial parts to the roots (Blakeslee et al. 2005).

Therefore polar transport of auxin is essential for the initiation and maintenance of polarized plant growth (Blakeslee et al. 2005; Carrier et al. 2008; Wang.S et al. 2011). The polar transport is based on chemiosmotic model (Goldsmith et al. 1981). Due to acid pH at extracellular space, around 5.5, the anionic form of auxin has to be protonated to be uptake inside the cell. Once in intracellular space, pH is higher around 7 and auxin can freely diffuse. To be able to leave the cell auxin ions needs active transport which use electrochemical gradient across plasma membrane (PM). Therefore polar transport of auxin requires both influx and efflux carriers (Goldsmith et al. 1981; Blakeslee et al. 2005; Carrier et al. 2008; Friml 2010). Thanks to genetic screens AUXIN RESISTANT 1/LIKE AUX1 (AUX/LAX) proteins have been identified as influx auxin carriers, PINFORMED (PIN), PIN-LIKES (PILS), and ATP-binding cassette transporters/multi-drug resistance/P-glycoprotein (ABCB/PGP) as efflux auxin carriers (Bennett et al. 1996; Palme and Gälweiler 1999; Noh et al. 2001).

II.1 Structure and function of auxin transporters

II.1.1 AUXIN RESISTANT 1/LIKE AUX1 (AUX1/LAX)

AUX1 was identified by a genetic screen of seedlings insensitive to the auxin 2.4-dichlorophnoxyacetic acid (2.4 D) (Bennett et al. 1996). It is a polytopic membrane protein composed of 11 transmembrane spanning domains, with N- and C-terminal hydrophilic domains oriented on cytoplasmic and apoplastic faces of the PM respectively (Swarup et al. 2004). AUX1 is similar to amino acid permeases and is found in all plant species analyzed to date (Petrásek et al. 2002; Schnabel and Frugoli 2004; Yang et al. 2006; Kerr and Bennett 2007). The function of AUX1 in mediating auxin transport has been analyzed both in plant through the study of *AUX1* mutant (Marchant et al. 1999) and in heterologous system using

Xenopus oocytes (Yang et al. 2006; Kerr and Bennett 2007). AUX1 is an influx carrier of auxin at its anionic form which coexists at the extracellular space with uncharged auxin form (Figure 6). The binding between AUX1 and IAA occurs specifically at apoplasm pH, between 5 and 6. When pH becomes higher than 7 the interaction is mainly reduced (Yang et al. 2006). AUX1 displays a non-polar membrane distribution and is also accumulated in Golgi and reticulum endoplasmic compartments (Kleine-Vehn et al. 2006). AUX1 allows the move of shoot auxin to the phloem for transport toward the root tip. It also mediates its transport out of lateral root (LR) cap at the root apex (Swarup et al. 2001; Swarup et al. 2004). Therefore AUX1 is localized at the lower ends of the cells in the lateral root cap, below the elongation zone in epidermal cells, columella and protophloem (Kleine-Vehn et al. 2006) (Figure 3).

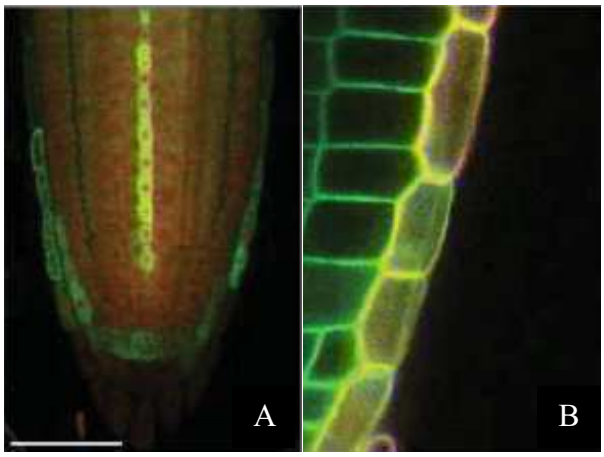

Figure 3: (A) Immunolocalization of the AUX1-YFP fusion protein (green and yellow) in transgenic Arabidospsis *AUX1* root, background is stained with propidium iodide (red). (B) Localization of AUX1-YFP fusion in Arabidopsis *aux1* lateral root cap cells (yellow) (Swarup et al. 2004 modified).

In arabidopsis AUX/LAX family is composed by AUX1 and three LAX members which are all able to transport auxin but in a tissue specific manner (Swarup et al. 2004; Bainbridge et al. 2008). For example the LAX3 protein has a function more in early stages of LR formation (de Billy et al. 2001; Swarup et al. 2008).

II.1.2 PINFORMED (PIN) family

The first *PIN* family member identified was called *PIN1*. Its function was determined in Arabidopsis through the analysis of loss-of-function mutant which presents altered vascular patterning, organogenesis and phyllotaxis. *PIN1* mutant fails to develop floral organs properly and generates pin-like inflorescences which gave the name PINFORMED family (Gälweiler et al. 1998; Reinhardt 2005; Krecek et al. 2009). In Arabidospsis eight *PIN* family members have been identified and six of them have been functionally characterized: *PIN1* (Gälweiler et al. 1998), *PIN2* (Müller et al. 1998; Utsuno et al. 1998), *PIN3* (Friml et al. 2002a), *PIN4* (Friml et al. 2002b), *PIN5* (Mravec et al. 2009) and *PIN7* (Friml et al. 2003). Their function as auxin efflux carrier protein was studied using Arabidopsis, tobacco BY-2 cells, human Hel1 or yeast cell cultures systems (Petrásek et al. 2006; Blakeslee et al. 2007). PIN proteins are predicted to display 10-12 transmembrane domains (Gälweiler et al. 1998; Blakeslee et al. 2007). In Arabidopsis PIN members can be divided in two subfamilies, short and long PINs, according to the presence of a central hydrophilic loop separating hydrophobic transmembrane domains. The subfamily of long PINs comprised PIN1, PIN2, PIN3, PIN4, PIN6 and PIN7 while PIN5 and PIN8 consist in short PINs subfamily (Petrásek et al. 2006; Carraro et al. 2006; Mravec et al. 2009). Long PINS are localized in PM in particular faces of the cell determining the localization of intracellular auxin flow (Wisniewska et al. 2006). Each long PIN protein displays tissue specific localization (Figure 4 and Figure 6). PIN1 is found in xylem parenchyma and participates to auxin transport from shoot to root tip. It mediates organogenesis and vascular tissue differenciation (Benková et al. 2003; Reinhardt 2005; Blakeslee et al. 2007; Grunewald and Friml 2010) (Figure 4). PIN2 presents a basal and lateral localization in root cortical cells and an apical localization in root epidermal cells allowing the redirection of auxin at the root tip. It is predominant in root gravitropical growth

(Chen et al. 1998; Müller et al. 1998; Grunewald and Friml 2010) (Figure 4). PIN3 is localized in root columella cells allowing auxin movement upon gravistimulation and at the inner surface of hypocotyls redirecting auxin into the vascular cylinder and in epidermal cells (Friml et al. 2002a; Grunewald and Friml 2010) (Figure 4). PIN4 is present in provascular quiescent centre and in daughter cells. It allows the determining of root meristem patterning (Friml et al. 2002b; Grunewald and Friml 2010) (Figure 4). PIN7 is abundant in epidermal tissues and involved in early embryo development stage allowing the establishment of the apical-basal axis particularly in the hypophysis (Friml et al. 2003; Blakeslee et al. 2007; Grunewald and Friml 2010) (Figure 4). Despite specific expression patterns of PIN proteins some redundancy could be observe between members. For example in *pin1* and *pin2* mutants, over-expression of other PIN homologues was sufficient to rescue normal auxin transport of the mutants (Vieten et al. 2005). Moreover, while PIN7 is thought to have a preponderant function during embryo development, *pin1,3,4,7* quadruple mutant displays stronger embryo lethal phenotype than single mutants (Friml et al. 2003).

Short PINs, PIN5 and PIN8, are mostly localized in endoplasmic reticulum (ER). They mediate auxin flow between cytoplasm and ER lumen which regulates subcellular auxin homeostasis (Mravec et al. 2009; Bosco et al. 2012). The ^{3}[H]-NAA retention experiments realized in BY-2 tobacco cells showed that PIN5 and PIN8 display distinct auxin transport activities. Indeed, the induction of *PIN5* expression resulted in no modification in NAA rate while thus of *PIN8* resulted in significant decrease in NAA retention (Ganguly et al. 2010). *PIN8* displays a distinct expression patten, being the only *PIN* family member accumulated in pollen (Bosco et al. 2012).

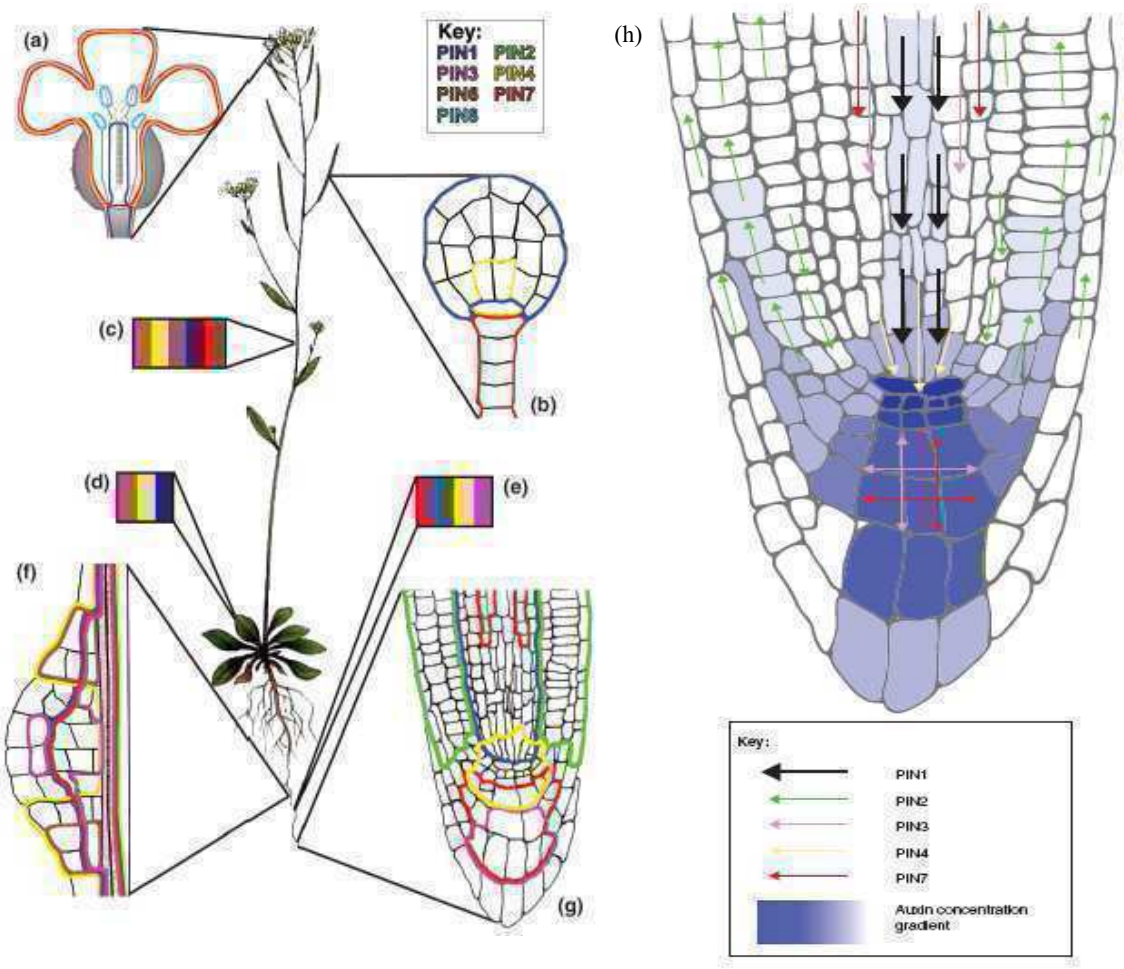

Figure 4: Expression map of Arabidopsis *PIN* genes compiled from both promoter activity data and protein localization. Each *PIN* gene expression domain is marker out by a colored line. The organs depicted are (a) flower, (b) embryo (late globular stage), (c) stem, (d) rosette leaf, (e) mature part of the primary root, (f) lateral root primordium stage 5, (g) root tip (h) Auxin distribution and PIN-dependent auxin-transport routes in the Arabidosps root tip. Auxin distribution (depicted as a blue gradient) has been inferred from DR5 activity and IAA immunolocalization. The localization of PIN transporters is based on immulocalization studies and on *in vivo* observations of proteins tagged with GFP. Arrows indicate auxin flow mediated by a particular PIN transporter (Krecek et al. 2009 modified).

II.1.3 PIN-likes (PILS) efflux transporters

More recently an *in silico* screening of proteins with predicted topology similar to PIN proteins has allowed the identification of a novel auxin transporters family called PILS. Despite that PILS proteins display low sequence identity with PINs (10-18%) they both present an InterPro auxin carrier domain. In Arabidopsis seven members of PILS family have been identified. Using transient expression of PILS-GFP reporter gene fusion it was established that PILS transporters are located on the ER membrane. Analysis of *PILS* Arabidopsis mutants and notably *pin2pin5* double mutant showed auxin-related phenotype, with notably altered hypocotyls and root growth. Moreover, using transient expression in tobacco, PILS protein negatively regulated the activity of the synthetic promoter DR5 containing AuxRE elements. These different results indicated that PILS negatively affect auxin signaling probably by affecting cellular auxin homeostasis (Barbez et al. 2012).

II.1.4 ABCB/multi-drug resistance/phospho-glycoprotein (ABCB/MDR/PGP) efflux transporters

The third class of auxin transporters is composed by PGPs that belong to the ABCB subgroup of the ATP-binding cassette (ABC) transporter family. In Arabidopsis, ABCB transporters are efflux auxin carriers which consist in a huge family (Figure 6). The best characterized members of ABCB proteins are ABCB1, ABCB4 and ABCB19 (Titapiwatanakun and Murphy 2009). Involvement of ABCB proteins in auxin transport was determined through the study of ABCB1 protein shown to be able to regulate hypocotyl elongation in a light-dependent manner (Sidler et al. 1998). ABCB19 displays very closed structure to ABCB1 (Verrier et al. 2008). ABCB19 was then shown to share similar function and expression with ABCB1 (Figure 5). Indeed the *ABCB19* mutant displays exaggerated phototropic and

gravitropic responses showing a function of ABCB19 protein in the repression of the light and gravity-stimulated hypocotyls growth (Noh et al. 2001; Lin and Wang 2005; Lewis et al. 2007; Wu et al. 2007; Nagashima et al. 2008). Moreover, double mutant *ABCB1ABCB19* exhibits stronger phenotypes than *ABCB1* and *ABCB19* single mutants (Noh et al. 2001). ABCB4 is involved in the movement of auxin away from the root tip and mostly regulates the export of auxin out of elongation zone (Figure 5). Indeed, *ABCB4* mutant exhibits decreased linear growth (Terasaka et al. 2005), increased rate of root bending (Lewis et al. 2007) and altered root hair development (Santelia et al. 2005; Cho et al. 2007). By contrast with *PIN* mutants none *ABCB* mutants display phenotypes in organogenesis suggesting that ABCB transporters are mainly involved in long-distance auxin transport instead of the establishment of basal auxin flows during organogenesis (Bandyopadhyay et al. 2007; Blakeslee et al. 2007; Bailly et al. 2008).

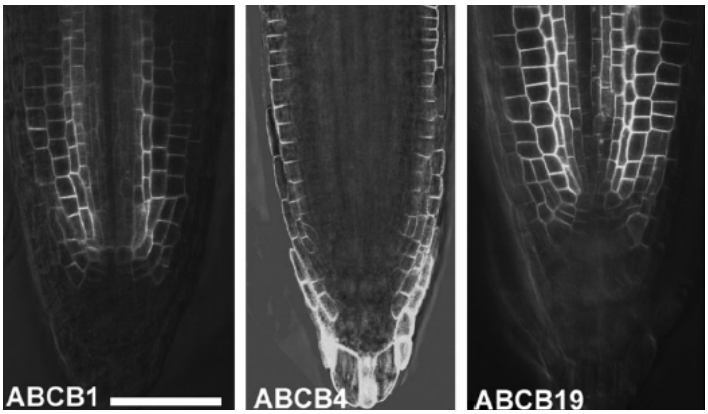

Figure 5: Localization of promoter-protein-GFP fusion for ABCB1, ABCB4, and ABCB19 proteins in Arabidopsis roots (Titapiwatanakun and Murphy 2009)

More recently it was demonstrated that ABCB14 and ABCB15 transporters are also involved in auxin transport but specific function remains not clear (Kaneda et al. 2011).

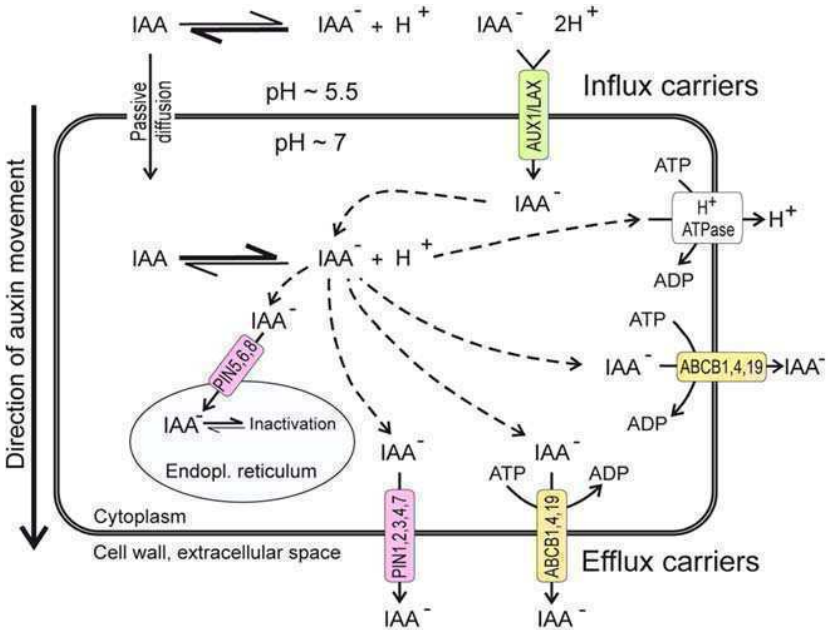

Figure 6: IAA transport (Friml 2010)

II.2 Regulation of auxin transporters

II.2.1 Regulation by auxin level

Numerous studies have shown that *PIN* expression and PIN protein abundance are altered by change in auxin levels (Peer et al. 2004; Vieten et al. 2005). Indeed endocytosis of PIN proteins had been shown to be inhibited by auxin (Paciorek et al. 2005; Zazímalová et al. 2007; Titapiwatanakun and Murphy 2009). Moreover, the inhibitors triiodobenzoic acid (TIBA) and pyrenoyl benzoic acid (PBA) both also disrupt PIN endocytosis by interfering with actin stability (Dhonukshe et al. 2008). The expression of *ABCB1*, *ABCB4* and *ABCB19*, is also upregulated by auxin (Noh et al. 2001; Geisler et al. 2005; Terasaka et al. 2005; Titapiwatanakun and Murphy 2009).

II.2.2 Subcellular trafficking

II.2.2.1 PIN trafficking

Subcellular trafficking of PIN1 is mediated by ADP-ribosylation factors (ARFs), the GNOM ARF guanine nucleotide exchange factor (ARF-GEF) and the ARF GTPase activating protein (ARF-GAP) SCARFACE (SFC) (Steinmann et al. 1999; Sieburth et al. 2006). GNOM regulation of PIN1 has been demonstrated by analysis of *GNOM* mutants whose embryos exhibit sever polarity defect and altered polar localization of PIN1 (Steinmann et al. 1999). GNOM protein could be regulated by the fungal toxin brefeldin A (BFA). Indeed treatment with BFA leads to intracellular aggregation of PIN1 in cells at the root apex, endocytotic vesicle being translocated from one side of the cell to the other. This BFA effect is no more observed when GNOM Sec7 domain is mutated (Geldner et al. 2001; Geldner et al. 2003; Kleine-Vehn and Friml 2008a; Kleine-Vehn et al. 2008b; Kleine-Vehn et al. 2008c). In addition, subcellular trafficking of PIN1 requires intact actin network but not microtubules (Geldner et al. 2001). While basal localization and endocytosis of PIN1 requires GNOM thus of PIN2 involves GNOMLIKE1 regulation (GNL1) which is resistant to BFA (Teh and Moore 2007; Kleine-Vehn et al. 2008c).

Apical localization of PIN2 is regulated by different mechanisms involving SORTING NEXIN1 (SNX1). SNX1 is a subunit of retromer complex which functions by recycling transmembrane proteins as PIN2 from endosomal multivesicular bodies (MVBs) to the trans-Golgi network (Bonifacino and Rojas 2006; Jaillais et al. 2006; Kleine-Vehn et al. 2008c). PIN1 could also be regulated by retromer complex. Indeed analysis of mutant in Vacualor Protein Sorting 29 (VPS29), one another subunit of retromer complex, showed altered PIN1 localization (Jaillais et al. 2007).

II.2.2.2 ABCB transporters trafficking

As for PIN2 endocytosis, ABCB19 trafficking involves GNL1. ABCB19 is more stable on plasma membrane and is not recycled by microtubule or SNX1-dependent processes. Nevertheless, treatment with BFA leads to aggregation of some part of ABCB19 protein in an unidentified compartment, showing that ABCB19 trafficking is also mediated by other component than GNL1. ABCB1 is less stable than ABCB19 and endocytosed following a BFA treatment suggesting that its trafficking involves GNOM (Titapiwatanakun and Murphy 2009). ABCB4 is stable in plasma membrane, like ABCB19, and its endocytosis is mediated by SNX1-endosome like for the apical localization of PIN2. Moreover ABCB4 is BFA-sensitive and can be regulated by GNOM but not by GNL1 (Cho et al. 2012)

II.2.2.3 AUX1/LAX transporters trafficking

Although AUX1 trafficking is also sensitive to BFA, it involves a GNOM-independent mechanism (Kleine-Vehn et al. 2006; Boutté et al. 2007). The localization of AUX1 on the plasma membrane of protophloem and epidermal cells requires the presence of AUXIN RESISTANT4 (AXR4) protein which is found in the ER. Indeed *AXR4* mutant presents abnormal accumulation of AUX1 in the ER of epidermal cells but does not affect PIN1 and PIN2 localization, suggesting that altered gravitopism phenotype is mainly due to defective AUX1 trafficking in the root epidermis (Dharmasiri et al. 2006).

II.2.3 Protein phosphorylation

To be active, PIN proteins have to be dephosphorylated. Therefore, the trimeric serine-theronine protein phosphatase 2A (PP2A) can positively regulates PIN activity by allowing their dephosphorylation (Michniewicz et al. 2007). Moreover, PIN proteins are also regulated by phosphorylation in an indirect manner. Indeed, the control of PIN polar localization by the

serine-threonine kinase PINOID protein requires phosphorylation of PINOID protein by 3-phosphoinositide-dependent protein kinase 1 (PDK1) (Christensen et al. 2000; Friml et al. 2004; Zegzouti et al. 2006).

Analysis of ABCB proteins structure revealed three possible sites in ABCB proteins than can be phosphorylated by related protein kinases (Nühse et al. 2004). The plasma membrane serine-threonine protein kinase PHOTOTROPIN1 (PHOT1) that is implied in multiple blue-light responses has thought to be potential candidate for ABCB phosphorylation (Inoue et al. 2008). Recently PHOT1 was shown to phosphorylate ABCB19 transporter, inhibiting its auxin efflux carrier activity (Christie et al. 2011).

II.2.4 Ubiquitin-mediated proteolysis

Protein ubiquitination generally leads to their degradation by the proteasome. Therefore protein ubiquitination regulates their concentration in tissue. After treatment with the proteasome inhibitor MG132, PIN1 and PIN2 are not degrataded any more suggesting that the concentration of these proteins is regulated by ubiquitination (Abas et al. 2006). Analyses of ABCB proteins activity in mouse have revealed that MG132 treatment also leads to an increase in ABCB proteins concentration suggesting that as PINs, ABCB can also be regulated by ubiquitination (Ogura et al. 2011).

II.2.5 Flavonoids

Flavonoids are able to negatively regulate polar auxin transport by down-regulating *PIN* genes expression and modifying subcellular localization of PIN1, PIN2 and PIN4 proteins (Murphy et al. 2000; Peer et al. 2004; Peer and Murphy 2007). Flavonoids are also able to inhibit ABDB-mediated auxin transport, notably ABCB4 (Peer and Murphy 2007).

II.2.6 Protein-protein interactions

Phenotypes of *FKBP immunophilin-like protein TWD1/FKBP42* mutant are similar to thus of *ABCB1ABCB19* double mutant (Geisler et al. 2003). It was then shown that TWD1/FKBP42 can activate ABCB1 and ABCB19 by interacting with their C-terminal domains (Bailly et al. 2008). Moreover because localization of ABCB1/19 remains unchanged in *TWD1* mutant, TWD1/FKBP42 seems not to be able to regulate ABCB transporters localization (Titapiwatanakun and Murphy 2009).

Subcellular colocalization, co-immunoprecipitation and yeast two-hybrid studies have shown that ABCB19 and PIN1 proteins can interact through their C-terminal part (Blakeslee et al. 2007; Bandyopadhyay et al. 2007). Co-immunoprecipitation analysis revealed that ABP1 and PIN1 could also interact but yeast-two hybrid experiment indicated that this interaction cannot be direct (Blakeslee et al. 2007).

II.2.7 Membrane composition

The sterol composition of plasma membrane is another important factor for PIN localization. Indeed mutant lacking STEROL METHYLTRANSFERASE1 (SMT1) which is required for first step of sterol biosynthesis displays aberrant cell polarity, auxin distribution, embryo development and altered PIN1 and PIN3 localization (Willemsen et al. 2003). Moreover, mutant in *cyclopropylsterol isomerase1 (CPI1)* that catalyses the step following SMT1 in sterol biosynthesis presents altered PIN2 localization (Men et al. 2008).

III Auxin perception

Auxin is recognized by numerous binding proteins which can be divided in two groups, soluble and non soluble proteins. During the last thirty years lot of biochemical studies were

investigated and allowed the identification of numerous auxin-binding proteins such as 1,3-glucanase (Macdonald et al. 1991), ß-glucosidase (Moore et al. 1992), glutathione S-transferase (Bilang et al. 1993) and superoxidedismutase (Feldwisch et al. 1995). The recognition of auxin by these proteins permits the initiation of further signal transduction chains, resulting in a specific physiological response. Each of these proteins displays different affinity with auxin and specific localization (Löbler and Klämbt 1985). The first auxin-binding protein described was called AUXIN BINDING PROTEIN 1 (ABP1) (Hertel et al. 1972; (Löbler and Klämbt 1985; Löbler and Klämbt 1985).

III.1 ABP1 pathway

III.1.1 Structure of ABP1

The ABP1 protein was isolated from maize coleoptiles membranes (Hertel and Thompson 1972). It is a small glycoprotein easily solubilized by detergents present on the outer leaflet of the ER membranes (Napier et al. 2002). The ABP1 structure is composed of conserved domains, a BoxA which allows the binding with auxin, a BoxB and a C-terminal KDEL tetrapeptide (Lys-Asp-Glu-Leu) which targets the protein to the ER (Hesse et al. 1989; Pelham 1989; Brown and Jones 1994) (Figure 7). The auxin-binding of ABP1 by a deep and hydrophobic pocket leads to a conformational change of the C-terminal part of the protein (Woo et al. 2000; Woo et al. 2002; Bertosa et al. 2008). ABP1 is encoded by a small gene family which member number and chromosome localization varies according to plant species (Palme et al. 1992; Shimomura et al. 1993; Watanabe and Shimomura 1998; Shimomura et al. 1999). In Arabidopsis ABP1 is encoded by only one gene (Palme et al. 1992).

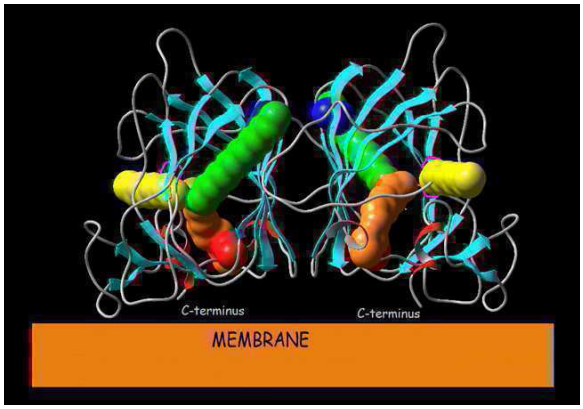

Figure 7: Orientation of the ABP1 homodimer to the membrane (Bertosa et al., 2008)

III.1.2 Function of ABP1

First studies showed a function of ABP1 in rapid regulation of membrane potential and potassium channels, inducing a shift in the cytosolic pH by a flux of potassium (Barbier-Brygoo et al. 1989; Barbier-Brygoo et al. 1992; Thiel et al. 1993; Gehring et al. 1998). It was then shown that the over-expression of *ABP1* leads to a highest sensitivity of guard cells to auxin (Bauly et al. 2000). Analysis of ABP1 function has been difficult due to the embryo lethality of homozygous knockout lines (Chen et al. 2001b) (Figure 8).

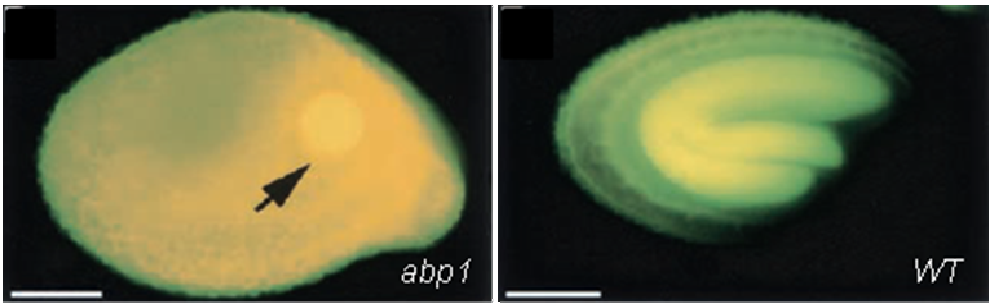

Figure 8: Globular embryo of the *abp1* mutant and WT at the same developmental age (Chen et al., 2001b modified)

Nevertheless over-expression of *ABP1* in leaves of Arabidopsis and tobacco showed the function of this protein in positively regulating cell elongation and division (Chen et al.

2001a; Chen et al. 2001b). More recently it was postulated that ABP1 could bind and activate calcium permeable ion channels (Shishova and Lindberg 2010). Moreover, analysis of heterozygous *abp1/ABP1* mutants which present an altered root development and gravitopism revealed that auxin-binding by ABP1 inhibits the endocytosis of auxin transporters PIN1, PIN2 and PIN3 (Robert et al. 2010; Effendi et al. 2011) (Figure 9). Nevertheless, the way by which ABP1 can modulate the clathrin machinery and therefore regulates PIN endocytosis remains to be understood (Robert et al. 2010). In addition, auxin binding by ABP1 coordinates the cytoskeleton structure by regulating the activity of two GTPases ROP2 and ROP6 (Xu et al. 2007; Sauer and Kleine-Vehn 2011).

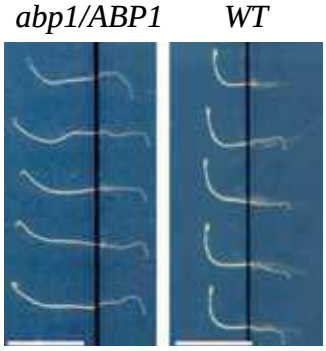

Figure 9: Gravitropic response of the hypocotyls of dark-grown 4-day-old seedlings of *abp1/ABP1* mutant and WT (Effendi et al., 2011 modified)

III.1.3 ABP1 pathway

Due to the absence of a transmembrane domain in ABP1 it is thought that the intracellular signal transduction mechanisms in response to auxin perception is mediated by one or more docking proteins (Klämbt 1990; Barbier-Brygoo et al. 1992). Indeed, it had been shown that proteins interacting with ABP1 at the external face of the ER membrane cannot fulfill this function (Shimomura 2006). Different models and putative docking proteins had been

proposed. Because of the enhancement of protein phosphorylation under auxin treatment, the docking protein is thought to have a protein kinase activity (Veluthambi and Poovaiah 1986). In another model, ABP1 form a G-protein coupled receptor with docking proteins (Macdonald et al. 1994; Hooley 1998). In this case, when auxin is bound to ABP1, the protein is able to activate the phospholipase A2 (PLA2) which transfers the signal (Scherer and André 1989; Scherer 1994; Scherer 2002; Scherer et al. 2007; Scherer 2011). One of putative docking protein is the receptor kinase FERONIA which can interact with G-proteins (Duan et al. 2010; Kanaoka and Torii 2010; Cheung and Wu 2011). Nevertheless, the complex formed between ABP1 and docking proteins remain to be determined. In conclusion, numerous responses to auxin in plants are mediated by ABP1 which are resuming in Figure 10.

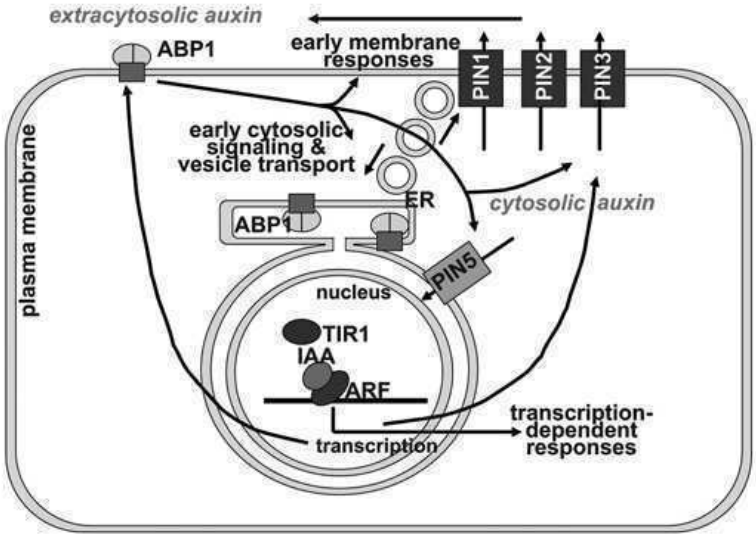

Figure 10: Model of auxin signal transduction (Effendi & Scherer, 2011).

III.2 S-PHASE KINASE-ASSOCIATED PROTEIN 2A (SKP2A) pathway

The SKP2A is a F-box protein which have been identified in Arabidopsis based on sequence similarity with human SKP2 (del Pozo et al. 2002). It regulates two cell division factors, the adenovirus E2 promoter binding factor C (E2FC) and the dimerization protein B (DPB) (del

Pozo et al. 2006; Jurado et al. 2008). Moreover, transcriptional factor E2FC is also involved in metabolic pathways and light signaling (de Jager et al. 2009). The SKP2A protein is post translationnally regulated by ubiquitination and therefore targeted to the proteasome (Jurado et al. 2008). More recently it had been shown that SKP2A presents an auxin-binding site in an hydrophobic pocket involving the Leu-128 residue (Jurado et al. 2010). Therefore the targeting to the proteasome and degradation of SKP2A is mediated by auxin-binding. Moreover, in absence of auxin-binding SKP2A is not able to regulate E2FC and DPB expression suggesting that interaction between these proteins occurred through auxin-binding site (Jurado et al. 2010). In conclusion auxin could be able to regulate cell division and maybe other pathways through SKP2A pathway.

III.3 TIR1 and Auxin receptor Fbox (AFB) paralogs pathway

III.3.1 Structure of TIR1 and AFBs

Determining of auxin perception mechanism was mainly improved by the discovery of the auxin soluble receptor, transport inhibitor resistant1 (TIR1) protein (Dharmasiri et al. 2005; Kepinski and Leyser 2005). This protein is part of F-box protein family and forms SCF (SKP1-Cullin-F-box) protein complex with SUPPRESSOR OF KINETOCHORE PROTEIN 1 (SKP1 or ASK1 in plants), CULLIN1 (CUL1) and RING BOX1 (RBX1) (Deshaies 1999; Skowyra et al. 1999; Cardozo and Pagano 2004) (Figure 11). SCF complexes consist in the largest multi subunit family of ubiquitin-ligase E3 in plants (Hershko and Ciechanover 1998; Gagne et al. 2002). In F-box proteins the F-box domain is localized near the amino terminal part and allows the binding with SKP1 (or ASK1) protein (Cardozo and Pagano 2004). The core of the SCF complexes is formed by the CUL1 and RBX1 subunits. CUL1 subunit binds SKP1 (or ASK1) and F-box protein through is amino terminal part and RBX1 subunit, which

recruits the E2-ubiquiting conjugating enzyme, by its carboxyl terminal part (Cardozo and Pagano 2004; Gilkerson et al. 2009).

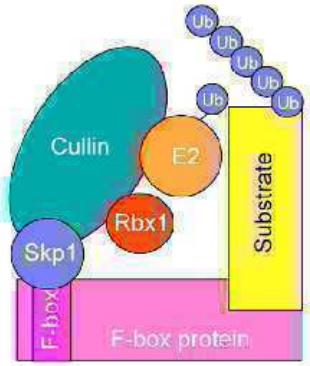

Figure 11: SCF complex (Hermand 2006)

The function of CUL1 and RBX1 subunits in SCF complex has been determined through the analysis of mutants as *axr1* or *axr6* which display strong phenotypes (Leyser et al. 1993; Hellmann et al. 2003; Gilkerson et al. 2009). In the case of auxin, the F-box protein present in SCF complex which allows its binding with auxin is TIR1. The interaction with auxin does not lead to a conformational change of the SCF^{TIR1} complex showing that unlike most hormones, auxin does not alter the shape of its receptor to modulate its activity (Shishova and Lindberg 2010). The carboxyl and amino terminal parts of auxin form two hydrogen bonds with TIR1 site at the residues Arg-403, Ser-438 and Leu-404, Leu-439 respectively (Shishova and Lindberg 2010).

In Arabidopsis, TIR1 has five homologs called AFBs (AFB1-5) which form related F-box proteins (Dharmasiri et al. 2005b). The closest related AFB to TIR1 is AFB1 with 70% of identity while AFB2-3 and AFB4-5 display only 60% and 50% identity respectively with TIR1. Moreover, due to the presence of an amino-terminal extension in AFB4-5 these proteins form a distinct group (Dharmasiri et al. 2005b; Parry et al. 2009).

III.3.2 Function of TIR1 and AFBs

III.3.2.1 TIR1 and AFB1-2-3 paralogs

Comparison of single and multiple mutants in TIR1 and AFB1, AFB2, AFB3 showed function of each of these proteins as auxin receptors (Dharmasiri et al. 2005b) (Figure 12). Nevertheless AFB1 and AFB2 proteins cannot replace TIR1 function in *tir1* mutant, even under the control of *tir1* promoter, showing the importance of TIR1 protein structure in auxin-binding (Parry et al. 2009).

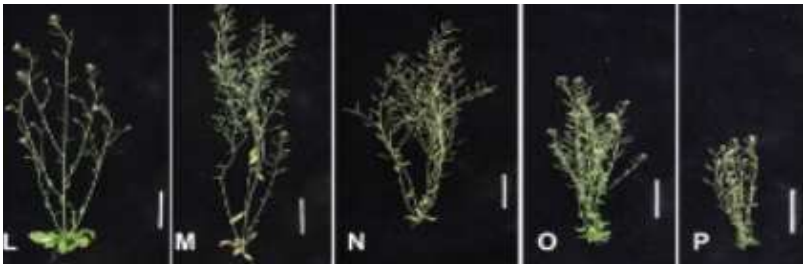

Figure 12: Phenotype of 40-day-old Col-0 (L), Ws (M), *tir1 afb2* (N), *tir1 afb2 afb3* (O), and *tir1 afb1 afb2 afb3* (P) plants. (Dharmasiri et al. 2005b)

However analysis of different mutant combinations showed a tissue specific function of TIR1 and AFB1-2-3 proteins, AFB1 protein being present in all tissues in seedlings but TIR1 and AFB2-3 being only localized in root tips, leaf primordial and shoot meristem. Therefore TIR1 and AFB2 seem to be more important for root development than AFB1 and AFB3 (Parry et al. 2009).

III.3.2.2 AFB4 and AFB5

AFB4 and AFB5 proteins display distinct structure and function in auxin perception. These two proteins are sensitive to the synthetic auxin picloram (4-amino-3,5,6-trichloropicolinic acid) studied for its auxinic herbicidal properties, contrary to TIR1 and AFB1-2-3 receptors (Walsh et al. 2006; Greenham et al. 2011). Analysis of *afb4* and *afb5* single or double mutants

revealed role of these receptors in hypocotyl and petiole elongation but also in root elongation and lateral root formation. While *afb5* mutant is similar to wild type, hypocotyls and petiole elongation are more altered in *afb4afb5* double mutant compared to single *afb4* mutant, suggesting that AFB5 should have a similar role to AFB4 but a less significant function. The *afb4* mutant phenotypes are opposite to thus of *tir1afb2* mutants. Moreover the *tir1afb2afb4* triple mutant displays same petiole and hypocotyl elongation than *afb4* single mutant showing that AFB4 is epinastic to TIR1 and AFB2 in these tissues and may act as a negative regulator of auxin response (Greenham et al. 2011; Hu et al. 2012) (Figure 13).

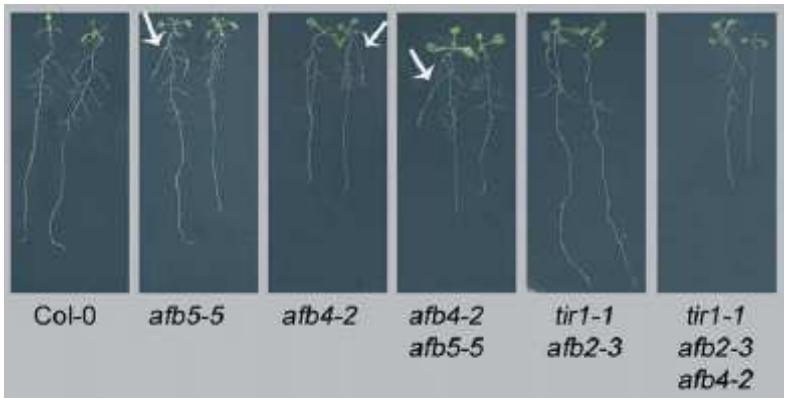

Figure 13: Phenotype of Col0, afb4, afb5 single mutant, *tir1afb2* double mutant and *tir1afb2afb4* triple mutant (Greenham et al. 2011)

III.3.3 TIR1 and AFBs pathway

Numerous studies have shown that TIR1 and AFBs paralogs are able to mediate auxin signaling by binding with auxin/indole-3-acetic acid (Aux/IAA) proteins (Kepinski and Leyser 2004; Dharmasiri et al. 2005a; Kepinski and Leyser 2005; Dharmasiri et al. 2005b). Aux/IAA proteins can interact with TIR1 and AFB proteins through a conserved domain called domain II composed by a hydrophobic motif GWPPV (Zenser et al. 2001; Tan et al. 2007) (Figure 14).

Figure 14: GWPP motif present in Aux/IAA conserved domain II (Tan et al. 2007)

Aux/IAA proteins bind closely to auxin-binding site and GWPPV motif is packed directly against auxin (Figure 15).

Figure 15: Auxin-TIR1-Aux/IAA interaction (Tan et al. 2007)

The Aux/IAA recognition does not lead to a conformational change of SCF^{TIR1} complex but needs TIR1-ASK1 interaction and is enhanced by auxin binding (Tan et al. 2007; Calderón Villalobos et al. 2012). Aux/IAA proteins display a much stronger interaction with TIR1 and AFB2 than with AFB1 and AFB3 F-box proteins. Moreover auxin-binding and complex formation is dependent on the nature of Aux/IAA proteins (Calderón Villalobos et al. 2012). The interaction between TIR1/AFB, auxin and Aux/IAA leads to the targeting of Aux/IAAs to the 26S proteasome and therefore to their degradation. This mechanism allows the auxin regulation of auxin response genes. This part will be more detailed in further section.

III.3.4 Regulation by the microRNA 393 (miR393)

Numerous studies have demonstrated that TIR1 and AFB1-2-3 paralogs (TAAR) could be regulated by a microRNA called miR393. The miR393 is produced from two loci called

miR393a and miR393b. The transcription of miR393 from these two loci is tissue-specific. For example in leaves, mir393 is mainly produced from miR393b locus. (Navarro et al. 2006; Parry et al. 2009; Si-Ammour et al. 2011; Windels and Vazquez 2011; Chen et al. 2011). The involvement of miR393 in TAAR regulation was determined by the analysis of *miR393* over-expression mutants which display in particular growth conditions auxin-resistant phenotype (Navarro et al. 2006; Parry et al. 2009). When plant is not able to produce miR393 the auxin perception by TAARs is increased which leads to altered leaves and cotyledons development (Parry et al. 2009). It is well known now that microRNAs regulate transcriptional expression of genes by cleaving thanks to site recognition on these genes (Djuranovic et al. 2012). Site recognition for mir393 has been found in all TAAR receptors. The putative regulation of miR393 by cleaving of TAAR was improved by the analysis of mutant with a miR393-resistant form of TIR1 which displays enhanced auxin sensitivity, inhibition of primary root growt, overproduction of lateral roots, altered leave structure and delayed flowering (Chen et al. 2011). More recently it has been determined that, in Arabidopsis leaves, miR393 produced from miR393b loci triggers the production of siRNAs from TAAR transcripts called siTAARs which have multi site cognition in all TAARs. Nevertheless this regulation pathway is restricted to the leaves. Other studies have shown that for example in roots, in presence of nitrate, miR393 directly cleaves *AFB3* transcripts but not *TIR1* and *AFB1-2* paralogs (Vidal et al. 2010). Moreover when leaves are infected by bacteria, TAAR cleavage is mediated by miR393 produced from miR393a loci instead of mir393b (Navarro et al. 2006).

IV Auxin signaling

One of the more significant features of the mode of action of auxin is the very quick change in regulation of auxin-responsive genes set in answer to a change in auxin concentration. Indeed

a modification in global gene expression can be observed within 5 to 15 min after an auxin application (Abel et al. 1994) and the change in protein level is detectable from 15 min after (Oeller and Theologis 1995). Therefore auxin signaling requires very efficient and quick pathway which actually involves two multigenic families, Aux/IAA and ARF genes.

IV.1 Aux/IAA

Aux/IAA genes have been identified thanks to the rapid induction of their expression in response to auxin (Abel et al. 1994; Abel et al. 1995). They form one of the three groups of primary-auxin-responsive genes with *Small Auxin Up RNA (SAUR)* and *Gretchen Hagen 3 (GH3)* (Theologis et al. 1985; Oeller et al. 1993). *Aux/IAA* genes have been found in many plant species but not in bacteria, animal or fungi and are probably unique to plants. All *Aux/IAA* members have been identified in Arabidopsis, maize, rice, poplar, sorghum and tomato. Their identification was performed notably thanks to sequence similarity with already known *Aux/IAA* genes and by yeast-two hybrid assays with Aux/IAA proteins. Indeed, Aux/IAA proteins display four conserved regions called domains I-IV, characteristic of this family (Reed 2001).

IV.1.1 Aux/IAA structure

Transient transformations of carrot protoplasts with *Aux/IAAs* from soybean, pea and Arabidopsis have shown the ability of Aux/IAA proteins to repress the activity of auxin-responsive reporter genes (Ulmasov et al. 1997; Tiwari et al. 2001). The repressor activity of Aux/IAAs was then shown to be mediated by domain I at their amino-terminal part constituted by conserved leucines (LxLxLx motif). Moreover the repressor activity of domain I is dominant over activation domains, whether if the activation domain is present as an intramolecular or intermolecular domain (Tiwari et al. 2004). This domain is similar to the so-

called EAR (Ethylene-responsive element binding factor-associated Amphiphilic Repression) repression domain found in some ERFs and SUPERMAN (Ohta et al. 2001; Hiratsu et al. 2003; Kagale et al. 2010; Kagale and Rozwadowski 2010; Kagale and Rozwadowski 2011). Nevertheless while the presence of alanine amino acid following the first leucine in EAR motif is critical, the nature of amino acid between leucines is not essential for Aux/IAA domain I (Ohta et al. 2001; Tiwari et al. 2004). It has been shown that the presence of two more leucines in AtIAA12 domain I (LxLxLxLxL) confers to the protein a stronger repressor activity (Hiratsu et al. 2003). Recent studies showed that some Aux/IAA proteins notably AtIAA7, AtIAA17, AtIAA14 and AtIAA16, display an additional domain I, a DLxLxL motif, that can also confers a repressor activity to the protein (Li et al. 2011a; Li et al. 2011b). Despite of conferring repressor activity to Aux/IAA domain I also allows the interaction between Aux/IAA and the C-terminal to lissencephaly homology (CTLH) domain of Topless (TPL) co-repressor (Szemenyei et al. 2008). The TPL family is constituted by five members in Arabidopsis, TPL and four TPL-related (Long et al. 2006). These genes were first described as direct interactors with WUSCHEL gene in Arabidopsis (Kieffer et al. 2006). The ability of Aux/IAA domain I to recruit TPL was first shown with AtIAA12. In absence of auxin, Aux/IAAs recruit TPL to suppress the expression of auxin-responsive genes (Szemenyei et al. 2008).

Aux/IAA proteins display another conserved domain called domain II composed by a hydrophobic motif GWPPV. Analysis of transient expression in carrot protoplasts of Aux/IAA domain II fused to firefly luciferase (LUC) showed that the presence of this domain confers instability to the LUC (Ramos et al. 2001). Moreover half-lives of Aux/IAA proteins are very short, ranging in Arabidopsis from 6 to 80 min depending on the protein (Abel et al. 1994; Worley et al. 2000; Ouellet et al. 2001; Gray et al. 2001). Domain II mutations

(mutation of G or P in GWPP motif) in individual members of Aux/IAA family result in a decrease in auxin response and to a stabilization of the affected protein (Rouse et al. 1998; Nagpal et al. 2000; Reed 2001; Rogg et al. 2001; Fukaki et al. 2002; Tian et al. 2002; Tatematsu et al. 2004) (Figure 16). Therefore Aux/IAA domain II confers instability to Aux/IAA proteins. Indeed, as previously reported in section III.3.3, in presence of auxin, Aux/IAA proteins can interact with auxin receptors TIR1/AFB proteins through domain II (Zenser et al. 2001; Tan et al. 2007). This binding allows the rapid ubiquitin-mediated degradation of Aux/IAA proteins which are targeted to the proteasome (Zenser et al. 2001; Gray et al. 2001; Tiwari et al. 2001).

The two other conserved domains in Aux/IAA proteins are called domains III and IV. They are located in the carboxyl part of the protein. These domains are also found in ARF proteins and allow the formation of homodimers and heterodimers with either a second Aux/IAA or an ARF (Kim et al. 1997; Ulmasov et al. 1997; Ulmasov et al. 1999b).

Despite that the four conserved domains are characteristic to the Aux/IAA family, some members lack one or more of these domains and are called non-canonical. In particular, some members do not have a conserved domain II and are therefore unable to be recognized by TIR1/AFB proteins, suggesting that these Aux/IAA proteins may be involved in other auxin regulation process (Jain et al. 2006; Kalluri et al. 2007; Sato and Yamamoto 2008; Wang.S et al. 2010; Wang.Yet al. 2010; Audran-Delalande et al. 2012).

In addition to conserved domains, Aux/IAA proteins display putative nuclear localization signals (NLS). NLS are composed by both the bipartite structure of conserved basic doublet KR between domains I and II and basic amino acids in domain II, and the SV40-type NLS located in domain IV. Transient transformation in tobacco protoplasts of Aux/IAA proteins

fused to the reporter gene GUS showed that these proteins are well localized in nucleus (Abel et al. 1994; Abel et al. 1995).

IV.1.1 Aux/IAA function

Aux/IAA function has been mostly studied in Arabidopsis through the characterization of gain-of-function mutants (mutation in domain II). Indeed, mutant screens have resulted in the recovery of gain-of-function mutants for nine *Aux/IAA* genes, this family being composed by 29 members in Arabidopsis (Liscum and Reed 2002; Tatematsu et al. 2004) (Figure 16).

IAA18	VVGWPPVRS	SLR/IAA14	VVGWPPVRN
crane-1	R	*slr-1,-4*	S
crane-2	E	*slr-2*	S
iaa18-1	E	*slr-3*	A
AXR5/IAA1	IVGWPPVRS	AXR3/IAA17	VVGWPPVRS
axr5-1	S	*axr3-1*	L
		axr3-3	G
SHY2/IAA3	IVGWPPVRS	*axr3-101*	E
shy2-1,-2	S		
shy2-3	E	MSG2/IAA19	VVGWPPVCS
shy2-6	L	*msg2-1*	S
		msg2-2	R
AXR2/IAA7	VVGWPPVRN	*msg2-3*	L
axr2-1	S	*msg2-4*	L
BDL/IAA12	VVGWPPIGL	IAA28	VVGWPPVRS
bodenlos	S	*iaa28-1*	L

Figure 16: Domain II mutations in Arabidopsis Aux/IAA gain-of-function mutants (Uehara et al. 2008)

Analysis of *axr5/iaa1* Arabidopsis mutant showed an alteration of root elongation, lateral root formation, hypocotyl elongation, root and shoot tropisms, leaf morphology and inflorescence. The *axr3/iaa17* Arabidopsis mutant presents shorter primary roots and formation of adventitious roots with altered root gravitropism. Moreover, leaves are darker and smaller than wild type and plants are dwarf showing altered apical dominance (Leyser et al. 1996)

(Figure 17C). In the case of *axr2/iaa7* Arabidopsis mutant the roots present a normal length but fewer root hairs and exaggerated lateral root formation. Like for *axr3/iaa17* mutant plants are dwarf with agravitropic shoots (Wilson et al. 1990) (Figure 17D). More recent studies have revealed that *AtIAA17* can also inhibit the timing of floral transition under short days light conditions by negatively regulating expression of GA20ox1 and GA20ox2 genes (Mai et al. 2011). The *iaa28* Arabidopsis mutant displays a reduced apical dominance with short inflorescence stems but contrary to *iaa18* and *iaa17* mutants developed leaves display no phenotype. At the root level phenotype of *iaa28* mutant is similar to thus of *iaa18* mutant with a reduced lateral root formation (Rogg et al. 2001) (Figure 17E). The *slr/iaa14* Arabidopsis mutant displays no more lateral root formation and altered hypocotyl gravitropic response (Fukaki et al. 2002) (Figure 17F). The *msg2/iaa19* Arabidopsis mutant displays similar phenotypes with altered hypocotyl gravitropism and lateral root formation. Moreover, growing in dark, this mutant fails in maintaining apical hook (Tatematsu et al. 2004). The only *AtIAA* for which function was analysed by characterization of both gain-of-function and loss-of-function mutant is *AtIAA3*. Indeed, gain-of-function *shy2/iaa3* mutant showed a reduced lateral root formation and increased root hair number with way root curvature and slow root re-orientation. At the opposite loss-of-function mutant displayed increased lateral root formation, increased way root curvature and accelerated root reorientation (Figure 17G). Taken together these data indicated that *AtIAA3* is a regulator of root development. Moreover the *shy2/iaa3* gain-of-function mutant is dwarf with short inflorescence stems (Tian and Reed 1999). The *bodenlos/iaa12* Arabidopsis mutant fails in initiating root meristem during early embryogenesis showing a specific role of *AtIAA12* during this process (Hamann et al. 2002).

In conclusion in Arabidopsis, the unraveling of Aux/IAA function was almost exclusively achieved from the characterization of gain-of-function mutants. The absence of phenotypes in

loss-of-function mutant (except *shy2/iaa3*) revealed an important functional redundancy among Aux/IAA family members (Fukaki et al. 2002; Fukaki et al. 2005; Overvoorde et al. 2005; Fukaki et al. 2006; Uehara et al. 2008).

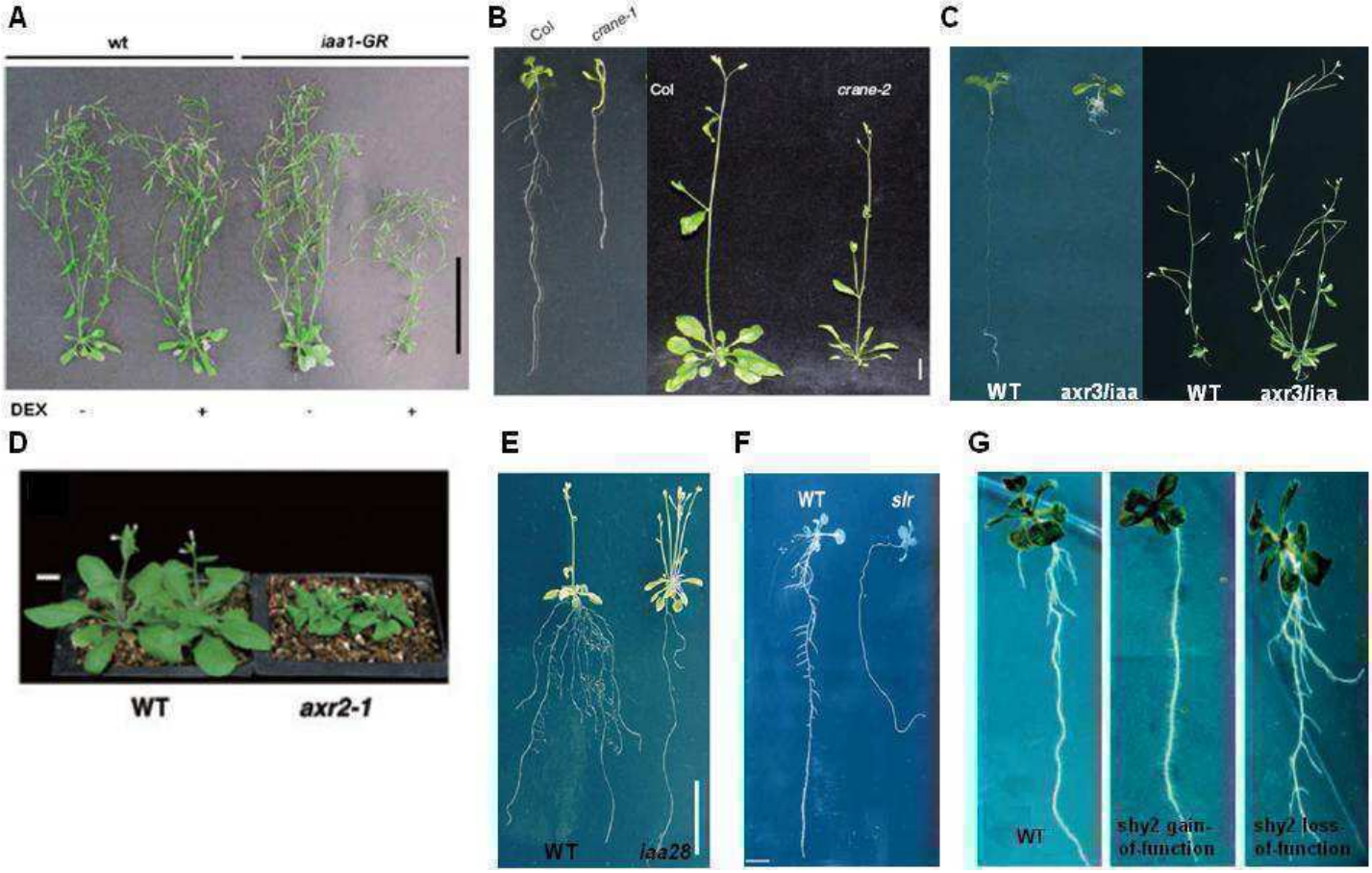

Figure 17: Aux/IAA gain-of-function Arabidopsis mutants phenotypes. (A) Wild type and *AtIAA1* transformed plants. *AtIAA1* expression was induced by DEX treatment (Park et al. 2002). (B) Wild type and *crane/iaa18* mutant phenotypes. Root development of fifteen-day old seedling (left) and one-month old plants (right) (Uehara et al. 2008). (C) Wild type and *axr3/iaa17* mutant phenotypes. Root development of fifteen-day old seedlings (left) and one-month old plants (right) (Leyser et al. 1996). (D) Wild type and *axr2/iaa7* mutant phenotype (Mai et al. 2011). (E) Wild type and *iaa28* mutant phenotype (Rogg et al. 2001). (F) Wild type and *slr/iaa14* mutant phenotypes (Fukaki et al. 2002). (G) Wild-type, *shy2/iaa3* gain-of-function and loss-of-function mutants phenotypes (Tian and Reed 1999).

IV.2 Auxin Response Factors (ARF)

IV.2.1 ARF structure

The ARF family is composed by 23 members in Arabidopsis (Guilfoyle et al. 1998; Liscum and Reed 2002). Most of ARF members display three conserved domains, an amino-terminal DNA binding domain (DBD) and domains III and IV at their carboxy-terminal part (Guilfoyle et al. 1998; Tiwari et al. 2003). The ARF DBD is a plant-specific B3-type domain, which is found in lot of plant transcription factors (Guilfoyle et al. 1998). The binding of ARF to DNA is facilitated by the presence of carboxy-terminal parts in ARF proteins (Ulmasov et al. 1999a; Tiwari et al. 2003). The ARF binding to DNA occurs by recognition of specific elements called AuxRE (TGTCTC or TGTCCC) which allow activation or repression of auxin-responsive genes (Ulmasov et al. 1999a; Ulmasov et al. 1999b).

The activity of ARF as activator or repressor is determined by the nature of their middle region. The influence of the nature of ARF middle region on their activity was demonstrated by fusing ARF middle regions to yeast GAL4 DBDs and testing the activity of these chimeric proteins on minimal promoter (Gus reporter genes with GAL4 DNA-binding domains). ARF with QSL-rich (glutamine, serine, leucine) middle region function as activator whereas thus with S-rich (serine), SPL-rich (serine, proline, leucine) and SL/G-rich (serine, leucine and/or glycine) middle region are repressor (Ulmasov et al. 1999a; Tiwari et al. 2003) (Figure 24). Due to the absence of middle region in AtARF23 its functional activity remains unknown (Figure 24).

The domains III and IV present at the carboxy-terminal part of ARF are related in amino acid sequence to thus present in Aux/IAA proteins. Therefore ARF can either form ARF

homodimers or Aux/IAA-ARF heterodimer. Nevertheless domains III and IV are absent in four AtARFs: ARF3, ARF13, ARF17 and ARF23, suggesting that these proteins cannot interact with Aux/IAAs (Guilfoyle et al. 1998; Liscum and Reed 2002) (Figure 24).

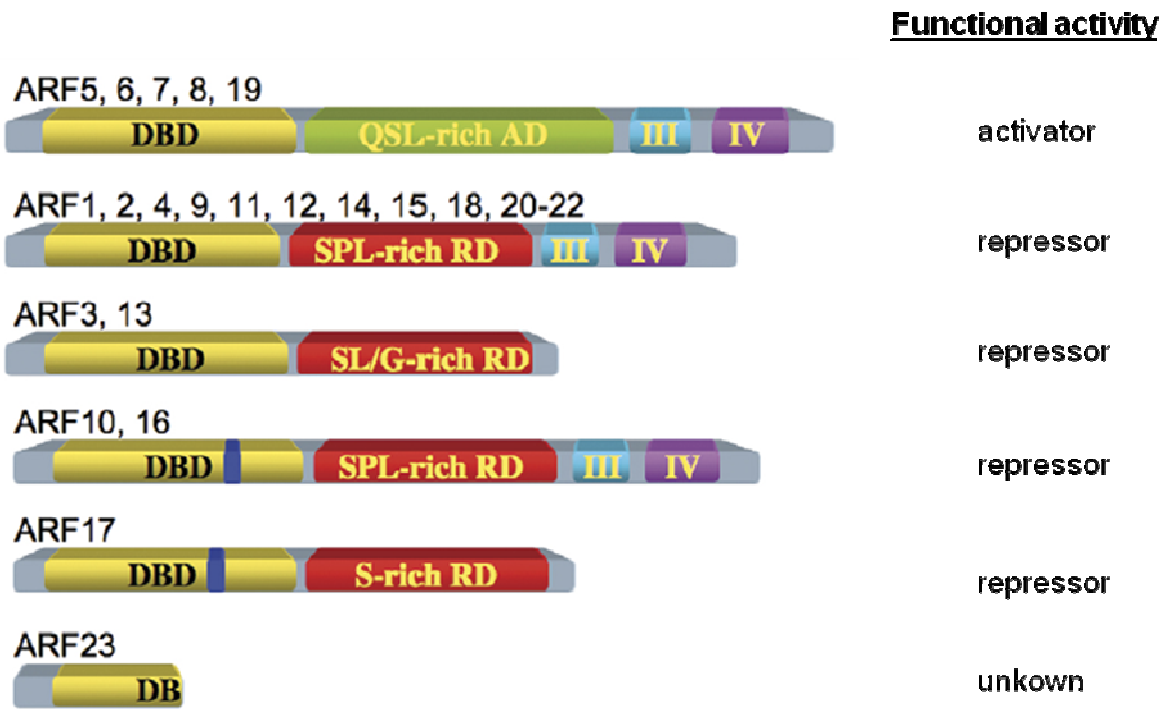

Figure 18: Structure of ARF family in Arabidopsis (Guilfoyle and Hagen 2007)

IV.2.2 ARF function

To determine putative function of ARF in Arabidopsis patterns of expression have been studied for many of them. These analysis showed specific expression pattern, *ARF1* and *ARF6* being mainly expressed in flowers (Nagpal et al. 2005; Ellis et al. 2005; Wu et al. 2006), *ARF2* in floral organs and seedlings (Okushima et al. 2005a; Ellis et al. 2005; Schruff et al. 2006), *ARF3* and *ARF4* in reproductive and vegetative tissues (Sessions et al. 1997; Pekker et al. 2005), *ARF5* in embryos and vascular tissues (Hardtke and Berleth 1998; Hardtke et al. 2004; Weijers et al. 2006; Wenzel et al. 2007), *ARF7* in roots, seedlings and embryos (Wilmoth et al. 2005; Okushima et al. 2007), *ARF8* in seedlings and flowers (Tian et al. 2004;

Nagpal et al. 2005; Goetz et al. 2006), *ARF12* in seeds (Okushima et al. 2005b), *ARF16* in roots, leaves, vascular tissues and embryos (Wang et al. 2005), *ARF19* in roots and seedlings (Okushima et al. 2005b; Wilmoth et al. 2005; Li et al. 2006). Moreover, lots of Arabidopsis mutants in one or more *ARF* genes have been studied.

The *arf2* Arabidopsis mutant displays delay in the induction of flowering, stamen elongation, floral organ abscission, silique ripening and rosette leaf senescence showing that *AtARF2* regulates transitions between multiple stages of development (Okushima et al. 2005; Ellis et al. 2005; Schruff et al. 2006; Lim et al. 2010) (Figure 19A). Moreover, *arf2* mutant presents larger inflorescence stems and ovule integuments due to an increase of cell number and size. Therefore ARF2 is able to inhibit cell division and elongation in ovules and stems (Okushima et al. 2005; Schruff et al. 2006). While *arf1* mutant shows no phenotype *arf1arf2* mutant presents same phenotypes than *arf2* single mutant but more marked suggesting that *AtARF1* acts redundantly to *AtARF2*. The *AtARF5* or *MONOPTEROS (MP)* gene is involved in formation of vascular tissues and in the initiation of the body axis in embryo. Indeed *arf5* mutant displays altered embryonic pattern with the incapacity to form apical-basal axis (Przemeck et al. 1996; Hardtke and Berleth 1998) (Figure 19B). The *AtARF7/NPH4* gene regulates leaf cell expansion, hypocotyls tropism and root development (Harper et al. 2000; Hardtke et al. 2004). Moreover it regulates leaf cell expansion and lateral root formation coordinately with *AtARF19*, *arf7arf19* double mutant displaying very few lateral roots and very small leaves (Wilmoth et al. 2005; Li et al. 2006; Okushima et al. 2007) (Figure 19C). The *arf3* Arabidopsis mutant, also known as *ETT*, displays abnormal development of flowers. Moreover, *arf3arf4* double mutant presents transformation of abaxial tissues into adaxial ones in all aerial parts, similar to thus of *KANADI* mutant, *KANADI* gene being involved in the establishment of abaxial identity (Figure 19D). Therefore, the expression of both *AtARF3* and

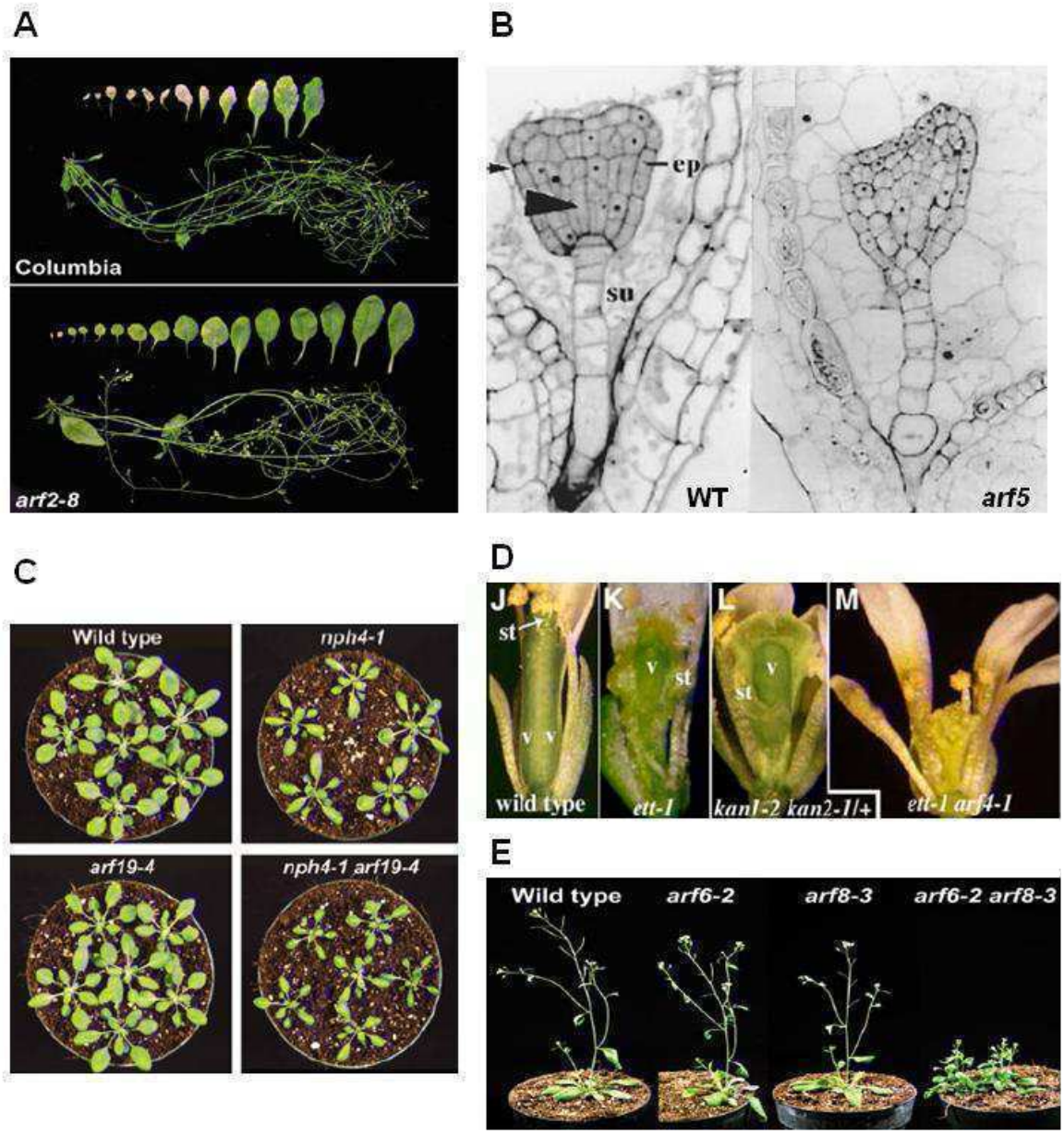

Figure 19: ARF Arabidopsis mutants phenotypes. (A) Wild type (columbia) and *arf2* mutant phenotypes. Natural rosette leaves senescence, inflorescence of 8-week-old plants (Ellis et al. 2005). (B) Embryos at heart-stage. *arf5* mutant displays no organized and elongated cells. Ep= epidermis, su = suspensor (Hardtke and Berleth 1998). (C) Rosettes of 33-day-old of wild type, *nph4*, *arf19* and *nph4/arf19* mutants (Wilmoth et al. 2005). (D) Flower development of wild type, *ett*, *kanadi* and *ettarf4* mutants (Pekker et al. 2005). (E) Phenotypes of 47-day-old wild type, *arf6*, *arf8* and *arf6arf8* mutants (Nagpal et al. 2005).

AtARF4 is required for specification of abaxial cells (Pekker et al. 2005). *AtARF6* and *AtARF8* both regulate flowering process by coordinating stamen development, petal expansion, anther dehiscence and gynoecium maturation. Indeed *arf6* and *arf8* single mutants display delayed flower maturation and reduced fertility but *arf6arf8* double mutant has complete arrested flower development before bud opening and are completely infertile (Nagpal et al. 2005; Reeves et al. 2012) (Figure 19E). While *arf10* and *arf16* single mutant present no phenotype the *arf10arf16* double mutant has root cap defects and abnormal root gravitropims showing a functional redundancy of these genes in regulating these processes (Wang.J et al. 2005).

IV.2.3 Regulation of ARFs

The absence of phenotypes in *arf10, arf16* and a*rf17* loss-of-function single mutants was further explained by the fact that these genes are negatively post-transcriptionally regulated by a micro-RNA (miR) called miR160. *ARF10,16 and ARF17* display site-recognition of *miR160* and indeed are cleaved by *miR160*. Mutant with *miR160*- resistant *ARF10, 16* and *ARF 17* forms, displays strong growth phenotypes and notably at embryo development level. Moreover, mutant with *mir160*-resistant *ARF10* form presents small narrow leaves and dies after juvenile stage (Mallory et al. 2005; Wang.J et al. 2005; Liu et al. 2007; Liu et al. 2010). Similarly, *miR167* targets and regulates *ARF6* and *ARF8*. Therefore the over-expression of *miR167* in Arabidopsis leads to similar phenotype to thus of *arf6arf8* double mutant. Mutant with *miR167*-resistant *ARF6* and *ARF8* forms presents also alteration of flower development with an arrested growth of ovule integuments and an alteration of anthers morphology. Therefore the control of *ARF6* and *ARF8* expression by *miR167* is essential for normal reproductive and floral development (Wu et al. 2006). In the case of *ARF2,3* and *ARF4*, they

are post-transcriptionally regulated by a trans-acting-small interfering RNAs (ta-siRNA) called TAS3 ta-siRNA. The formation of this ta-siRNA from primary transcripts involves miR390, RNA-DEPENDANT RNA POLYMERASE6 (RDR6) and DICER-LIKE4 (DCL4) (Williams et al. 2005).

IV.3 Protein interactions

Auxin signaling is mainly regulated by the interactions between Aux/IAA and ARF proteins. Indeed, the similar conserved domains III and IV present in these proteins allow the formation of Aux/IAA-ARF heterodimers. Therefore in the absence of auxin, Aux/IAAs can bind ARFs, preventing ARFs from activating the transcription of their target genes (Guilfoyle and Hagen 2007; Szemenyei et al. 2008). The presence of auxin promotes the association of Aux/IAAs to the SKP1-Cullin-F-box (SCF) complex through the binding to the auxin transport inhibitor response1 (TIR1) or to its paralogs AUXIN RECEPTOR F-BOX (AFB) proteins. The SCF complex targets Aux/IAAs to the proteasome leading to their rapid degradation (Dharmasiri et al. 2005a; Dharmasiri et al. 2005b; Kepinski and Leyser 2005; Leyser 2006; Tan et al. 2007; Chapman and Estelle 2009). The degradation of Aux/IAAs results in the release of ARFs which can then activate the transcription of target genes via binding to the Auxin Responsive Elements (AuxRE) present in the promoter regions of auxin-regulated genes (Hagen et al. 1991; Ulmasov et al. 1997; Hagen and Guilfoyle 2002).

In Arabidopsis protein-protein interactions between the 29 Aux/IAAs and 23 ARFs have been mostly studied through yeast-two hybrid experiment. These studies show that most of Aux/IAAs are able to interact with ARF activators while ARF repressors display very few interactions. To date, the only ARF repressor which is able to interact with different Aux/IAA genes is AtARF9. Therefore it is suggesting that ARF repressors may more act in a

competition for AuxRE element binding with ARF activators rather than be directly involved in auxin signaling through Aux/IAA regulation (Weijers et al. 2005; Vernoux et al. 2011). Moreover, the analysis of Aux/IAA and ARF mutants in Arabidopsis show putative *in planta* interaction between these two protein families. Indeed, AtIAA12 could interact with ARF5, regulating embryonic root formation (Weijers et al. 2005) and AtIAA14 specifically interacts with AtARF7 and AtARF19 inactivating lateral root formation (Fukaki et al. 2006).

Despite of Aux/IAA-ARF, the TPL-Aux/IAA interaction is also involved in the regulation of Aux/IAA signaling. Indeed most of Aux/IAAs in Arabidopsis have been shown to be able to interact with TPL or TPL-related proteins through their domain I. The TPL proteins play the function of co-repressors and it is suggested that Aux/IAA recruits TPL to strongly repress ARF activity (Szemenyei et al. 2008; Causier et al. 2012).

V Roles of auxin in plants

V.1 Organogenesis

V.1.1 Cell division

The role of auxin in cell division was shown by the study of cell culture. Indeed auxin starvation of cells leads to the arrest of cell division which can be restored by application of auxin (Stals and Inzé 2001; Inzé and De Veylder 2006). The cell cycle of division is divided in four phases: G1 phase, S phase (synthesis) during which DNA is replicated, G2 phase and M phase (mitosis). The G1 and G2 phases are two critical regulatory steps mainly regulated (Inzé and De Veylder 2006). The plant cell-cycle progression is regulated by mechanism involving cyclin-dependant kinase (CDK) and cyclin complexes (CYC) which phosphorylate substrates during G1 or G2 phases. Cell division occurs with the initiation of CYCD

transcription which then associates with CDKA to create an active complex. This complex inactivates the retinoblastoma tumor suppressor protein (RBR) by phosphorylation which leads to the release of the E2FA/B (adenovirus E2 promoter binding factor) and DP (dimerization protein) complexes (Figure 20). The E2FA/B-DP complex regulates the expression of genes required for entry into S-phase. Moreover, the CDKA-CYCD complex can also inactivate by phosphorylation the E2FC transcriptional repressor of cell division (Inzé and De Veylder 2006; Perrot-Rechenmann 2010) (Figure 20). Auxin was shown to be important for the assembly of active CDKA-associated complexes in tobacco cell suspension culture (Harashima et al. 2007).

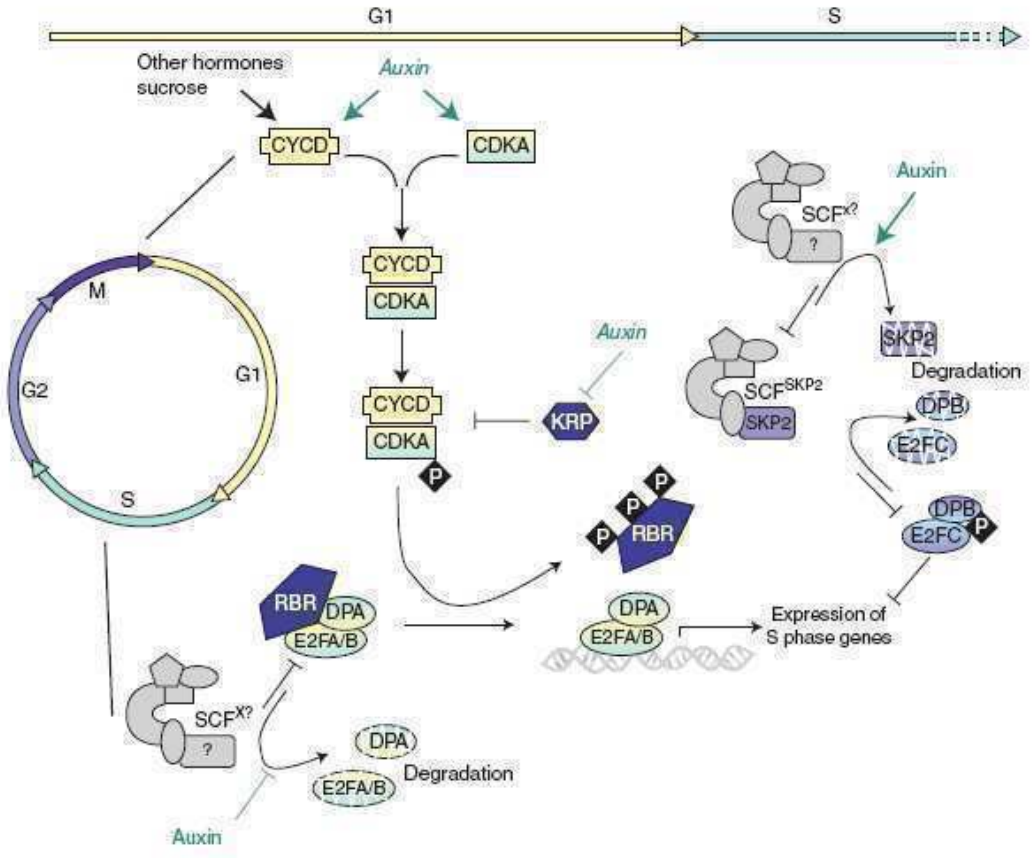

Figure 20: Auxin regulation of G1/S transition during cell division (Perrot-Rechenmann 2010)

The expression of CYCD is regulated by auxin (Dewitte and Murray 2003). Moreover, the activity of CDKA-CYCD complex can be repressed by Kip-Related Protein (KRP) whose expression is down-regulated by auxin (Richard et al. 2001; Himanen et al. 2002)

The auxin control of cell division is thought to be mediated by both ABP1 and SCF^{TIR1} pathways. Indeed, inactivation of ABP1 in tobacco cells leads to cell-cycle arrest in G1 phase (David et al. 2007) and the loss-of-function *ABP1* Arabidopsis mutant displays aberrant cell division in the suspensor during embryo development (Chen et al. 2001a). Moreover the characterization of *axr5/iaa1* mutant revealed that *AtIAA1* gene is involved in the regulation of cell division (Ku et al. 2009).

V.1.2 Cell expansion

After division, cells generally enlarge to involve of thousands of times their original size. Auxin was shown to induce rapid cell elongation in stem, coleoptile and hypocotyls segments (Rayle and Cleland 1992). The well known process allowing cell expansion is driven by the extension of cell wall and an increased internal turgor pressure due to water uptake. The activation of H^+ ATPases present on the plasma membrane leads to the hyperpolarization of the membrane potential and the activation of K^+ channels which allows water uptake (Ruck et al. 1993; Philippar et al. 1999; Philippar et al. 2004). Auxin activates the expression of both H^+ ATPases and K^+ channels (Philippar et al. 1999). Moreover to allow the cell wall extension new material has to be incorporated such as xyloglucan chains. This process is in part mediated by Xyloglucan endotransglucosylase/hydrolases (XTH) whose expression is up-regulated by auxin (Yokoyama and Nishitani 2006). In addition, expansines and b-glucanases also present in cell wall are up-regulated by auxin (Kotake et al. 2000; Swarup et al. 2008). The other process also controlling cell elongation is the increase in cell ploidy level thanks to

endoreplication but poor is known about this mechanism. Nevertheless auxin can modulate the stability of E2FC which in addition to be a negative regulator of cell division, is also necessary for endoreplication (del Pozo et al. 2006).

The auxin control of cell expansion is mainly mediated through ABP1 pathway. Indeed, over-expression of ABP1 leads to an increased expansion of some leaf tissues and the null Arabidopsis *ABP1* mutant displays altered cell expansion (Chen et al. 2001a; Chen et al. 2001b).

V.2 Root formation and development

Analysis of root development under different auxin concentration treatment showed that auxin is involved in the regulation of root growth. Although low auxin concentration stimulates root growth but at hight level auxin plays a function of root elongation inhibitor (Mulkey et al. 1982; Golaz and Pilet 1987). Therefore root elongation is strongly regulated by auxin gradient formed thanks to auxin transporter distribution among root (Friml et al. 2002; Krecek et al. 2009). The most studied aspect of root development is the lateral root (LR) initiation and development process. In Arabidopsis, LR are initiated by several anticlinal divisions of pericycle cell layers adjacent to the xylem pole to form an early-stage primordium (Laskowski et al. 1995; Malamy and Benfey 1997; Dubrovsky et al. 2001). The function of auxin in LR formation was determined by the characterization of Arabidopsis mutant. Indeed, Arabidopsis mutants *sur1* and *sur2* which present enhanced auxin biosynthesis, display increased lateral root formation (Boerjan et al. 1995; Delarue et al. 1998). At the opposite, Arabidopsis mutants *pin2* and *aux1* with deficient auxin transport present a reduction in LR formation (Marchant et al. 2002; Benková et al. 2003; Dubrovsky et al. 2009). Therefore, accumulation of auxin in pericycle cells lead to their transformation into LR founder cells which then divide

and form the primordium (Dubrovsky et al. 2008). Nevertheless when auxin level is too high, leading to the inhibition of primary root elongation, auxin also acts as an inhibitor of LR formation (Ivanchenko et al. 2010). Studies of Aux/IAA and ARF Arabidopsis mutant lead to the determination of different Aux/IAA-ARF modules regulating LR formation. Before LR intiation, module composed by AtIAA28 and unknown ARFs controls the LR founder cell specification which occurs in the root basal meristem (De Rybel et al. 2010). Then nuclear migration and asymmetric cell divisions of LR founder cells are regulated by SLR/IAA14-ARF7-ARF19 module (Okushima et al. 2005; Fukaki et al. 2005; Okushima et al. 2007; Goh et al. 2012a; Goh et al 2012b). Moreover, this process is also regulated by BDL/IAA12-MP/ARF5 which represses ectopic pericycle cell divisions (De Smet et al. 2010). In addition, SHY2/IAA3-ARFs positively regulate LR primordium development and in parallel inhibit LR formation by affecting auxin homeostasis (Goh et al. 2012a ; Goh et al 2012b) (Figure 21).

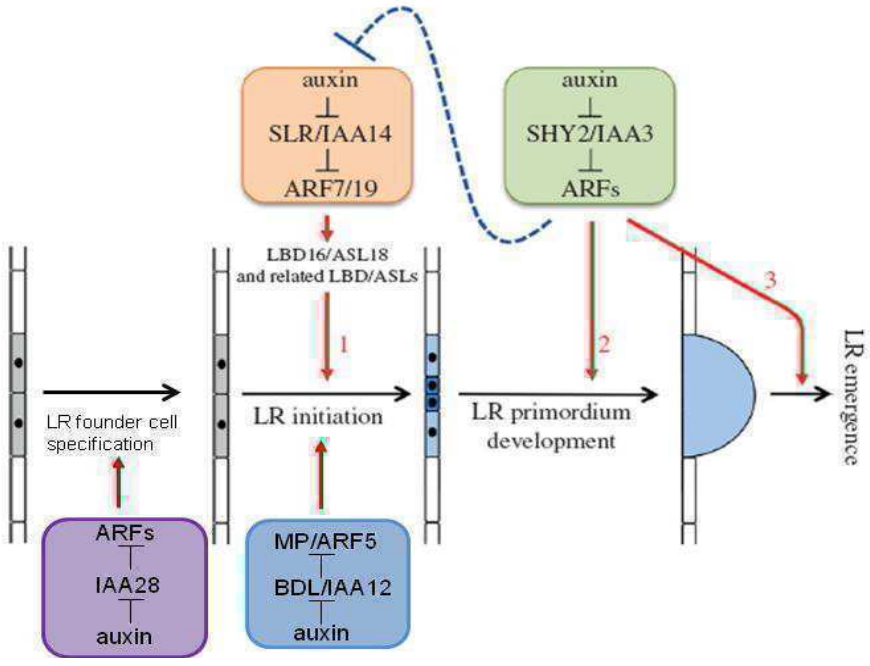

Figure 21: Schematic representation of Aux/IAA-ARF modules regulating lateral root formation (Goh et al. 2012)

In addition root gravitropism is controlled by auxin. Upon gravistimulation, auxin accumulation is modified into the root which leads to the formation of a lateral auxin gradient and therefore to a differential cell elongation (Boonsirichai et al. 2002). The modification of auxin local accumulation is mainly mediated by the AUX1 transporter (Marchant et al. 1999). The importance of auxin in mediating root gravitropism is underlined by the phenotype of *axr3/iaa17* Arabidopsis mutant which displays altered root gravitropism (Leyser et al. 1996).

V.3 Organ patterning

V.3.1 Vegetative development

During plant growth, organs are developed from the shoot apical meristem (SAM). The SAM controls both organ formation and phyllotaxis which is the arrangement of leaves on a stem. The presence of auxin within the L1 layer of the SAM (Figure 22) is essential for organ initiation (Heisler and Jönsson 2007) and the formation of lateral leaves is initiated at auxin accumulation sites (Reinhardt et al. 2000; Reinhardt et al. 2003). Therefore active auxin transport mediated by the PIN transporters is essential for the creation of auxin maxima which patterns organ development (Benková et al. 2003; Heisler et al. 2005; Scarpella et al. 2006; Petrásek et al. 2006). Indeed the Arabidopsis loss-of-function mutant *pin1* fails to initiate reproductive lateral organs and produces leaves with altered vascular tissue patterning (Okada et al. 1991; Gälweiler et al. 1998; Mattsson et al. 2003; Koizumi et al. 2005). In addition to regulate leaves formation auxin also controls leave structure and leaflet development.

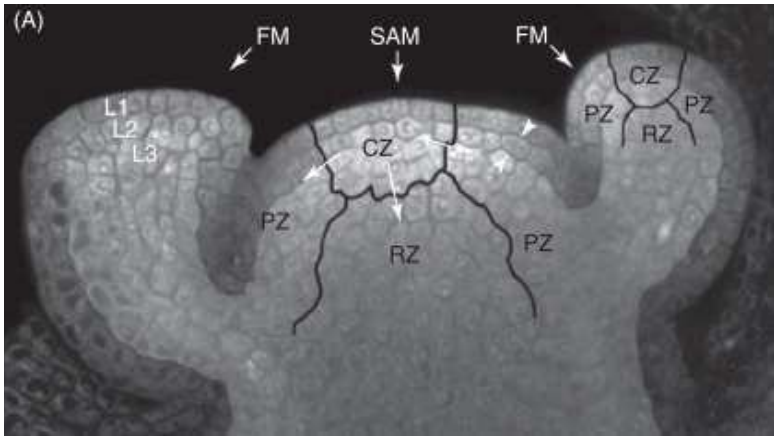

Figure 22: Organization of the Arabidopsis shoot apical meristem (SAM). Confocal laser-scanning micrograph through an inflorescence SAM and its adjacent floral meristems (FMs). Superimposed on the micrograph are the meristem layers and zones. The shaded domains depict the three cell layers: the epidermal (L1) layer, the subepidermal layer (L2), and the internal corpus layers (L3). The black outlines represent the approximate boundaries between the meristematic zones: the central zone (CZ), the peripheral zone (PZ), and the rib zone (RZ) (Ha et al. 2010).

Indeed, disruption of auxin transport results in leaf simplification, *pin1* mutant displaying simple leaves (Reinhardt et al. 2000; DeMason and Chawla 2004; Barkoulas et al. 2008). Moreover, the *iaa8iaa9* double knockout Arabidopsis mutant displays altered leave structure (Koenig et al. 2009) and the tomato loss-of-function *iaa9* develops simple leaves (Wang.H et al. 2005) (Figure 23).

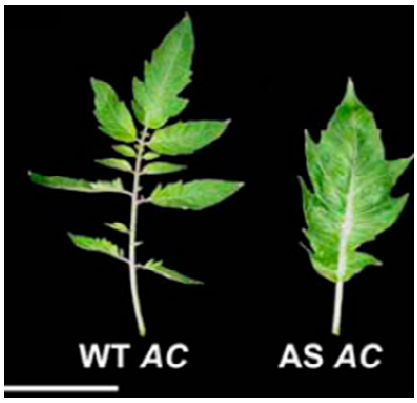

Figure 23: Tomato leaf morphology in wild-type Ailsa Craig (WT AC) and *IAA9* antisense (AS AC), Bar=10cm (Wang.H et al. 2005)

Auxin is also involved in leaf senescence. The auxin levels decline with leaf age to lead to leaf senescence when auxin level is equal between the leave and stalk (Shoji et al. 1951). Addition of auxin to the distal and proximal end of abscission zone explants leads to delayed and promoted abscission respectively (ADDICOTT and LYNCH 1951).

V.3.2 Floral development

Auxin also controls floral meristems (FM) which are initiated at the flanks of the central SAM (Figure 22). Similarly to SAM, FM control floral organs formation and spatial distribution (Cheng and Zhao 2007). Indeed high auxin concentration is observed in all young primordia and application of auxin to the FM leads to the formation of oversized flower primordia (Reinhardt et al. 2003; Aloni et al. 2006). Accumulation of auxin is also essential for anther development (Cecchetti et al. 2008) and is involved in stamen and pollen development. Mutants in auxin biosynthesis pathway (*yuc2yuc6*), in auxin perception (*tir1afb*) and auxin signaling (*arf6arf8*) all fail in elongating stamen filaments at anthesis stage (Nagpal et al. 2005; Cheng et al. 2006; Cecchetti et al. 2008). Alteration in auxin biosynthesis or transport both result in altered pollen development (Feng et al. 2006; Cheng et al. 2006). The presence of auxin is also necessary for apical-basal patterning of the gynoecium, *ett/arf3* and *arf5* Arabidopsis mutants displaying enlarged apical and basal regions with strongly reduced ovaries (Okada et al. 1991; Przemeck et al. 1996; Sessions et al. 1997). Taken together data indicate that high auxin level is required in apical region to promote style and stigma development while low level allows ovary and gynoecium formation (Nemhauser et al. 2000).

V.4 Fertilization and Fruit growth

As previously described auxin plays a crucial role in proper development of all reproductive organs. In addition, auxin prevents premature anther dehiscence and pollen maturation

allowing normal fertilization process. Alteration of auxin perception in *tir1afb* mutant results in premature anther dehiscence while application of auxin leads to the maintaining of anther (Cecchetti et al. 2004; Cecchetti et al. 2008). In addition, pollen of *tir1afb* mutant initiates earlier mitosis leading to precocious pollen maturation (Feng et al. 2006; Cecchetti et al. 2008). In addition to be essential for pollination, auxin allows fruit development in absence of fertilization. Indeed, application of auxin to emasculated flowers result in parthenocarpic fruit development (Gustafson 1936). Higher auxin production in carpel due to ectopic expression of Agrobacterium iaaM under the control of carpel specific promoter leads to the formation of parthenocarpic fruits. The auxin-resistant tomato mutant, *diageotropica*, displays reduced fruit set, fruit weight and seed production (Balbi and Lomax 2003). In Arabidopsis alteration of *AtARF8* expression leads to the formation of parthenocarpic fruits (Vivian-Smith et al. 2001; Goetz et al. 2006; Goetz et al. 2007). In the loss-of-function tomato *iaa9* mutant ovary develops prior to pollination leading to precocious fruit set and marker fruit parthenocarpy (Wang.H et al. 2005).

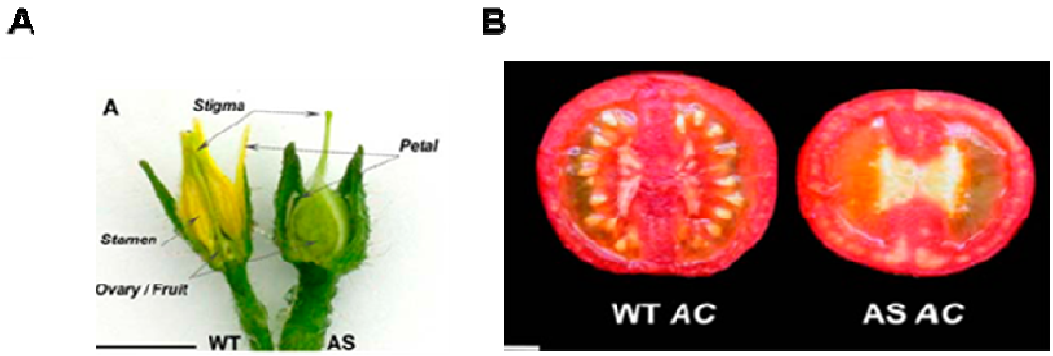

Figure 24: (A) Flower buds at 1 d before anthesis in wild-type and AS-IAA9 lines (AS), showing dramatically enlarged ovary and underdeveloped stamen in AS-IAA9 lines. (B) Wild-type Ailsa Craig seeded fruit (WT AC) and AS-IAA9 parthenocarpic fruits (AS AC). Bars = 10 mm (Wang.H et al. 2005).

While ethylene is known to be an essential regulator of fruit ripening, auxin seems also involved in this process. Indeed, ripening begins at the same time with decline in auxin levels and in auxin-regulated gene expression (Given et al. 1988; Cohen 1996; C Davies 1997; Catalá et al. 2000). Moreover application of auxin results in a delayed fruit ripening (Vendrell 1985; Manning 1994; C Davies 1997; Aharoni et al. 2002). The loss-of-function tomato *DR12* mutant, an ARF, displays altered fruit ripening with dark green, blotchy fruit and enhanced fruit firmness (Jones et al. 2002), suggesting that auxin is really involved in proper fruit ripening process.

VI Interaction between auxin and other hormones

VI.1 Auxin and cytokinin (CK)

CK is essential for whole plant development controlling either embryogenesis, organogenesis or root development. It interacts with auxin to regulate all of these processes. Indeed, CK controls auxin biosynthesis through positive regulation of *TAA1* and *YUCCA6* genes expression involved in IPA pathway and *CYP79B2*, *CYP79B3* and *NIT3* implied in IAOx pathway (Jones et al. 2010; Zhou et al. 2011). Moreover, auxin and CK display antagonist function during early embryogenesis. In the basal cell of the hypophysis auxin represses CK function by activating the expression of *type A Arabidopsis Response Regulator (ARR)*, *ARR7* and *ARR15*, which are CK signaling repressors (Müller and Sheen 2008) (Figure 25A). At organ patterning level, auxin and CK both regulate SAM activity. Indeed, CK deficiency at SAM level results in reduced SAM size and activity (Werner et al. 2003; Higuchi et al. 2004; Werner and Schmülling 2009). CK accumulates at the central zone of SAM while auxin is mostly present in peripheral zones suggesting that auxin down-regulates CK to trigger organ initiation (Jasinski et al. 2005; Yanai et al. 2005; Zhao et al. 2010). Moreover, auxin is

transported from the SAM to inhibit CK biosynthesis and axillaries bud growth (Nordström et al. 2004) (Figure 25B).

Auxin and CK also control root development, displaying antagonist function on cell division and differenciation in the root meristem. The cross-talk between auxin and CK in roots is mediated by *shy2/iaa3* (Figures 25C and 25D). Indeed CK application leads to up-regulation of *shy2/iaa3* expression in the vascular tissues of root meristem, this gene repressing ARF activity and therefore the regulation of cell division and differentiation mediated by auxin (Dello Ioio et al. 2007; Dello Ioio et al. 2008a; Dello Ioio et al. 2008b; Moubayidin et al. 2009; Goh et al. 2012a; Goh et al. 2012b). Moreover, CK inhibits the expression of several PINs in root meristem and in LR primordium and therefore prevents auxin accumulation required for normal root development (Figure 25C).

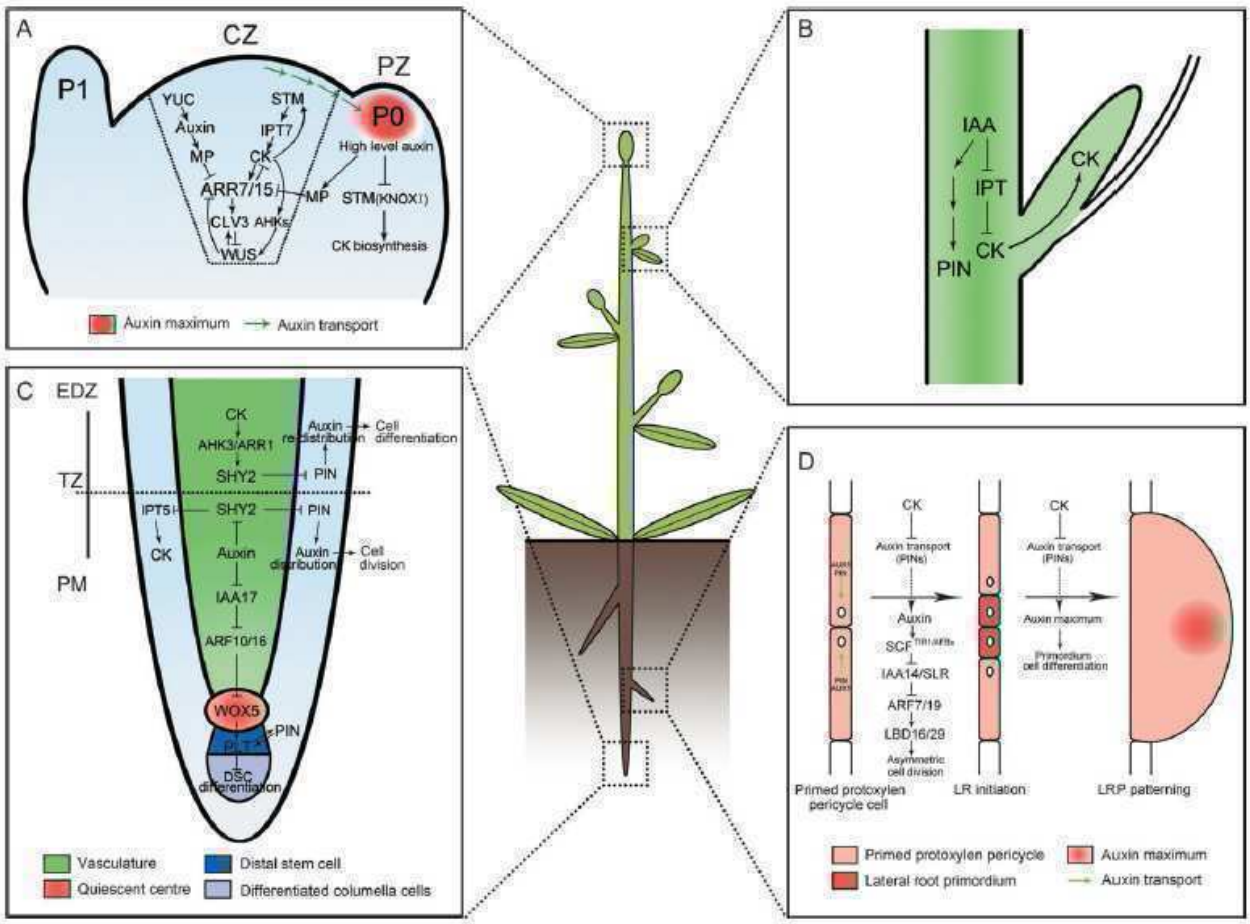

Figure 25 : Molecular mechanism of auxin and cytokinin interaction in the regulation of Plant Meristem Development. (A) Auxin repression of cytokinin in shoot meristem through regulation of *ARR7* and *ARR15*. (B) Auxin repression of cytokinin to inhibit axillary bud growth. (C) Auxin and cytokinin interaction at root meristem though SHY2/IAA3 regulation. (D) Auxin and cytokinin interaction in the regulation of lateral root initiation and development (Su et al. 2011).

VI.2 Auxin and brassinosteroids (BRs)

BRs play like auxin an essential role in controlling cell elongation. Long time ago it was shown that BRs promote cell elongation when supplied with auxin (Mandava 1988). Similarly, BRs treatment significantly enhances auxin response in hypocotyl elongation (Vert

et al. 2008). Mutants of genes involved in BRs or auxin pathways show similar phenotypes and notably dramatic dwarf phenotype (Halliday 2004; Teale et al. 2008). Moreover the auxin-responsive mutants *axr2/iaa7*, *iaa17/axr3*, *arf2* and *tir1* display reduced sensitivity to BRs and aberrant BRs-induced gene expression (Nemhauser et al. 2004; Nakamura et al. 2006; Vert et al. 2008). Similarly, expression of auxin transporters *PIN4* and *PIN7* is repressed in the BRs deficient mutant *det2* (Nakamura et al. 2004; Nemhauser et al. 2004; Li et al. 2005). The expression of *AtIAA5* and *AtIAA19* is induced by both auxin and BRs and, in addition, requires BRs biosynthesis (Nakamura et al. 2006). These different data show that BRs and auxin coordinately regulate plant development and particulary hypocotyl elongation and root development and that this cross-talk is mediated by notably the regulation of *Aux/IAA* expression (Nemhauser et al. 2004; Nakamura et al. 2006; Hardtke 2007; Teale et al. 2008). In addition it was shown that BIN2 (Brassinosteroid Insensitive 2) which is involved in BRs signaling can directly interact with ARF2. This interaction results in ARF2 phosphorylation and in a loss of ARF2 DNA binding and repression activities (Vert et al. 2008).

VI.3 Auxin and jasmonate (JA)

JA is known to positively regulate floral development but to inhibit LR formation. To promote floral development cross-talk between JA and auxin is essential. The *arf6arf8* double mutant displays a dramatic reduction in JA level in flowers and the abnormal development of stamen can be rescued by JA treatment. It was then shown that ARF6 and ARF8 can induce JA synthesis which, in turn, induces expression of *MYB21* and *MYB24*, these *MYB* transcription factors promoting petal and stamen growth (Nagpal et al. 2005; Cecchetti et al. 2008). Moreover JA and auxin display antagonist function in regulating LR development.

Indeed, JA down-regulates auxin transporters PIN1 and PIN2 protein levels which blocks auxin accumulation in the root basal meristem essential for LR formation (Sun et al. 2011).

VI.4 Auxin and strigolactone

Strigolactone is the most recently phytohorme identified. It is described as an important regulator of branching either in shoots or in roots. It was shown in pea, rice and Arabidopsis that the expression of genes involved in strigolactone synthesis, the *RAMOSUS* genes, *RMS*1 and *RMS*5, is regulated by auxin (Beveridge et al. 2000; Foo et al. 2005; Johnson et al. 2006; Arite et al. 2007; Hayward et al. 2009). Moreover, branching in *axr1* and *tir1afb* mutants was inhibited by strigolactone treatment. Application of strigolactone suppresses adventitious root formation in Arabidopsis and pea (Rasmussen et al. 2012). This suggested that auxin inhibits shoot branching and promotes adventitious root formation by down-regulating strigolactone synthesis (Beveridge et al. 2009).

VI.1 Auxin and Abscisic acid (ABA)

ABA is an important regulator of plant development and response to environmental stresses. At root level, low concentrations of ABA promote root growth while high concentrations inhibit root growth by blocking cell division (Himmelbach et al. 1998; Yin et al. 2009; Zhang et al. 2010). Therefore ABA promotes root growth in a similar way than auxin. A cross-talk between these two hormones at root level can be observed, the *axr2/iaa7* Arabidopsis mutant showing an ABA-insensitive phenotype to roots (Wilson et al. 1990; Nagpal et al. 2000). The expression of *AtARF2* is induced by ABA treatment and loss-of-function *arf2* mutant displays enhanced ABA sensitivity at root level. Moreover, ABA is also known to be a key regulator of seed germination. *Arf2* loss-of-function mutant also displays altered seed germination such as *axr1* and *axr2/iaa7* mutants. Actually, ARF2 negatively regulates the expression of a

homeodomain gene *HB33* whose expression is also reduced by ABA. Therefore auxin and ABA cross-talk at root level and in seed germination is mediated at least in part through ARF2 and HB33, playing respectively the function of negative and positive regulator of ABA signaling (Wang.L et al. 2011).

In addition, ABA and auxin display antagonistic function in the regulation of stomatal aperture. Auxin allows the opening of stomatal pore whereas ABA promotes its closure (Eckert and Kaldenhoff 2000).

VI.2 Auxin and ethylene

Auxin and ethylene interact at different levels in plant development but they display either synergistic or opposite effects. The two hormones are able to regulate the synthesis of each other. Ethylene synthesis involves consecutive action of three enzymes: S-adenosyl- L-methionine (SAM) synthase, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, and ACC oxidase. Auxin can induce the expression of eight out of the nine Arabidopsis *ACC synthase* genes, being involved in the conversion of SAM to ACC (Abel et al. 1996; Wang et al. 2002) (Figure 26). Reciprocally, ethylene regulates the expression of *WEI2/ASA1* and *WEI7/ASB1*, the subunits of an anthranilase synthase that catalyses the first step in tryptophane biosynthesis, the principal precursor of auxin biosynthesis (Stepanova et al. 2005).

At root level, as auxin, ethylene inhibits primary root growth and root hair formation but at the opposite of auxin ethylene also blocks LR formation (Swarup et al. 2002). Moreover, analysis of ethylene treatment effect in a variety of auxin genes related mutants showed that auxin biosynthesis transport and signaling are required to mediate ethylene inhibition of root growth (Stepanova et al. 2005; Stepanova et al. 2007).

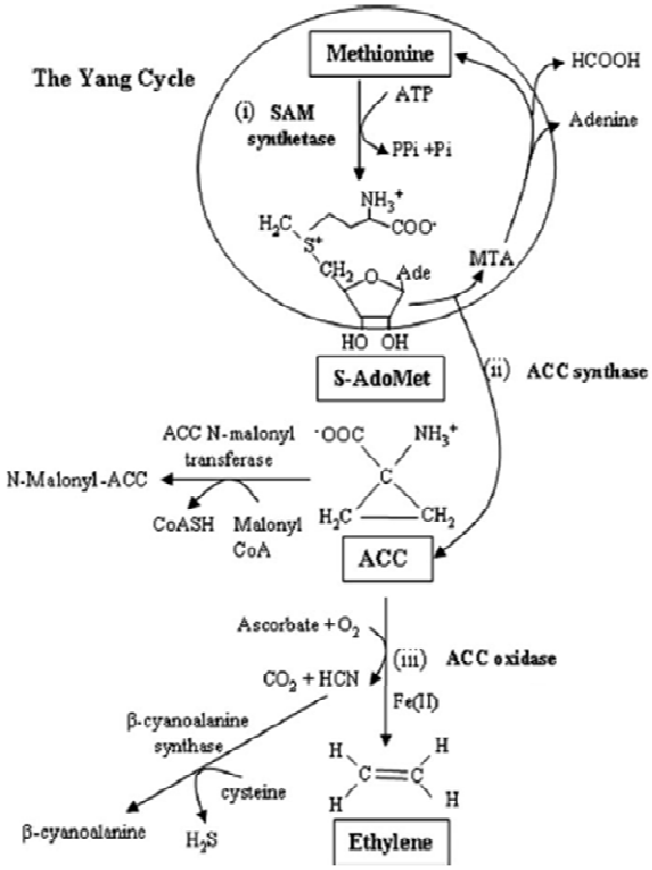

Figure 26: Ethylene biosynthesis pathway (Lin et al. 2009)

The induction of apical hook formation in Arabidopsis represents one of the best described examples of auxin-ethylene cross-talk in plants (Lehman et al. 1996; Raz and Ecker 1999). The bending (180°) of hypocotyls at apex zone, just below cotyledons, results in the formation of an apical hook. This is mediated by an asymmetrical accumulation of auxin which leads to differential cell elongation of opposite sides of the hypocotyls (Kuhn and Galston 1992; Lehman et al. 1996). Apical hook formation is also promoted by ethylene. Indeed ethylene treatment leads to the formation of exaggerated hooks and ethylene-

insensitive mutants are not able to form apical hook (Guzmán and Ecker 1990). Nevertheless ethylene action is mediated by change of expression of genes related to auxin pathway showing that ethylene requires auxin to control apical hook formation (Stepanova et al. 2008; Vandenbussche et al. 2010; Zádníková et al. 2010). In addition tomato transgenic plants where *Sl-IAA3* is down-regulated, display both auxin and ethylene related phenotype underlying the auxin and ethylene cross-talk notably in mediating apical hook formation (Chaabouni et al. 2009a; Chaabouni Salma et al. 2009b).

Auxin and ethylene both also control fruits and flowers abscission acting respectively as accelerator and repressor. Moreover, auxin and ethylene cross-talk is necessary to determine cell separation site. Indeed ethylene induces the formation of cell separation zone and auxin determines its localization (McManus et al. 1998).

VI.3 Auxin and GA

Gibberellin (GA) mediates regulation of stem development and hypocotyls growth. A number of studies have demonstrated that GA interacts with auxin to control these processes (Frigerio et al. 2006; Desgagné-Penix and Sponsel 2008; Stavang et al. 2009; O'Neill et al. 2010; Ross et al. 2011). It was shown in pea and Arabidopsis that auxin positively regulates in these tissues the *GA 20-oxidase (GA20ox)* and *GA3ox* genes involved in GA biosynthesis but also represses thus of *GA2ox*, an inactivator of GA biosynthesis (Ross et al. 2001; O'Neill and Ross 2002; Frigerio et al. 2006). Similarly auxin biosynthesis is regulated by GA through the control of the expression of *PIF4 (phytochrome interacting factor 4)* (Chapman et al. 2012). In addition GA biosynthesis is required for auxin-dependent hypocotyls growth, long hypocotyl phenotype of *YUCCA1* mutant being suppressed by the GA biosynthesis inhibitor paclobutrazol (Frigerio et al. 2006). In line with the regulation of hypocotyls growth GA also

regulates apical hook formation by modulating the expression of auxin transporters *PIN3* and *PIN7* (Achard et al. 2003; Alabadí et al. 2004; Vriezen et al. 2004).

In roots, GA promotes root elongation and inhibits LR formation. Nevertheless GA stimulation of root elongation requires auxin. In addition GA controls LR formation by regulating polar auxin transport (Gou et al. 2010).

In addition numerous studies have shown that auxin and GA both regulate fruit set in tomato. The increase of auxin content in ovaries leads to the formation of parthenocarpic fruit but this effect can be inhibited by application of paclobutrazol, an inhibitor of GA biosynthesis (Serrani et al. 2008; Serrani et al. 2010). The auxin regulation of fruit set is mediated by the degradation of Aux/IAA proteins which results in the activation of ARFs. One of ARFs, ARF7, have been shown to particularly regulate fruit set in tomato but also to be able to control expression of genes involved in GA signaling pathway (Teale et al. 2006; de Jong et al. 2011).

VII The tomato: a plant model

VII.1 An agronomic plant

The tomato *(Solanum lycropersicum)* is originated from South America and was imported in France during the sixteen century. Long time seen as toxical, the tomato was first cultivated during the eighteen century. From this date selection and cross between species were realized to create hybride varieties more adapted to culture. The first hybride variety developed for industrial culture was called “Roma”, in 1955. Since, most varieties cultivated for industrial purpose results from cross with Roma variety. The development of varieties with long conservation, good color and fruit quality and resistance to major diseases is a real agronomic

challenge. Indeed, to date, the tomato is the second most important vegetable crop cultivates after potato. The tomato world production is around 280 million of tones per year. (http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1210). They are around 7500 tomato varieties cultivated to date presenting a huge variability in tomato fruit color or size. In fact, tomato fruit size varies from 5mm of diameter in cherry tomatoes to more than 10 centimeters. While most cultivars produce red fruit, number of them display yellow, orange, purle, green or black fruit. In addition, tomato is used for many purposes in industry, either for direct consummation or to make sauces.

VII.2 A plant model

In addition to its agronomic importance the tomato displays several characteristics that make it a convenient model plant species for scientific studies. Indeed the tomato displays a relatively compact genome around 900 Mb divided in 12 chromosomes. A marker-saturated genetic linkage map (SGN database http://solgenomics.net), a rich germplasm collection (Tomato Genetics Resource Center http://tgrc.ucdavis.edu/), EMS mutant collections and TILLING (Targeting Induced Local Lesions IN Genomes) mutants (CFGB Bordeaux and URGV Evry) and RNAseq data (www.sgn.cornell.edu and www.tigr.org) are available. Moreover highly efficient transformation protocols have been performed allowing easy generation of tomato transgenic plants. In addition the tomato displays developmental traits not found in Arabidopsis such as photoperiod-independent sympodial flowering, compound leaves, glandular trichomes and the formation of fleshy climacteric fruits. The tomato is member of Solanaceae species and its study could allow improving of the knowledge also for other Solanaceae important agronomic plants such as tobacco, potato, pepper or eggplant. Recently the tomato genome has been sequenced allowing the easy identification in this

specie of genes involved in fruit development. The Genomic and Biotechnology of fruits laboratory participated mainly in the sequencing of the tomato genome. Comparison of tomato genome with thus of other Solanaceae such as pepper, eggplant and potato showed a high syntheny between those genomes. This result underlies the possibility to use tomato scientific data for the understanding of other Solanaceae species. Moreover comparison between grape genome and tomato and potato genomes showed that a more recent triplication occurred in tomato and potato which resulted in the adding of new gene family members important for fruit-specific function (The tomato genome consortium 2012). In addition, the tomato produces fleshy fruits for which ripening is told climacteric, meaning that it is regulated by an increase in respiration and in ethylene biosynthesis. Numerous agronomic species display climacteric fruit ripening, it is notably the case for apple, banana or avocado (Lelievre et al. 1997). During ripening, numerous processes undergo such as the increase in biosynthesis of carotenoids, flavonoids vitamins and flavor volatiles which determine final fruit quality. Lots of internal and external signals regulate fruit ripening such as hormones, light or temperature. Therefore understanding mechanisms that control these processes is essential to improve fruit quality. All together this allows the tomato to be an excellent plant model for studying fleshy fruit and climacteric fruit development and ripening.

VII.3 Plant characteristics

The tomato plant is a dicotyledon which grows as a series of branching stems. After the formation of principal inflorescence, lateral buds develop and form secondaries inflorescence. Most of tomato plants have compound leaves made up of five to nine leaflets which are distributed along the leaf rachis. Leaflets are connected to the leaf rachis by the petiolule and leaf is linked to the stem through the petiole (Figure 27B).

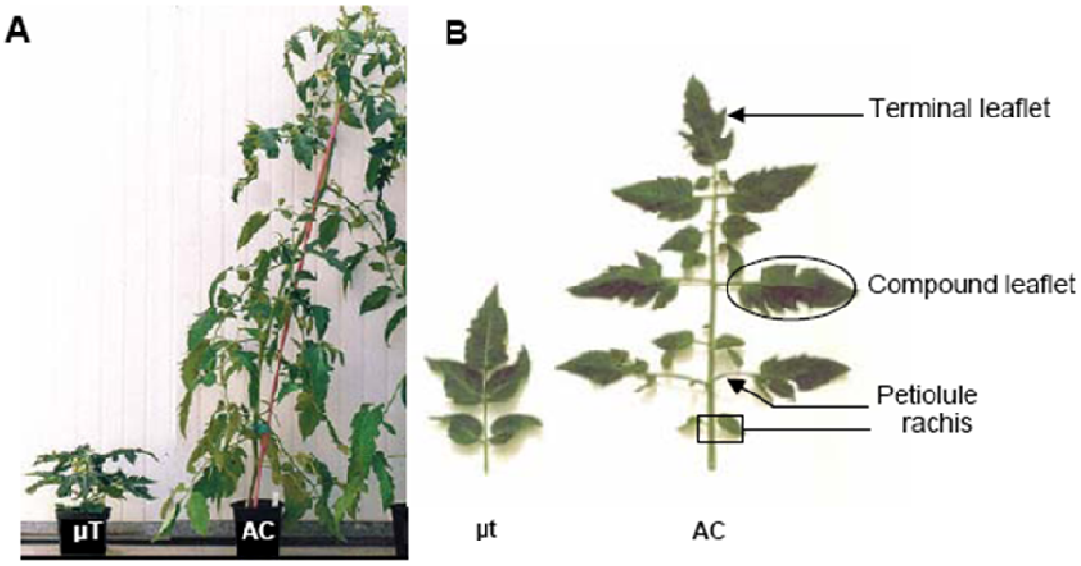

Figure 27: Plant of micro-Tom (µT), Ailsa Craig (AC). (A) Plants at the time of flowering. (B) Compound leaves.

Tomato leaf is composed by different tissues, epidermis on both abaxial and adaxial side, palisade mesophyll composed by parenchyma cells, spongy mesophyll constituted by irregurlaly shaped parenchyma cells and vascular tissues (xylem and phloem) (Figure 28). Photosynthesis principally occurs in the mesophyll part of the leaf.

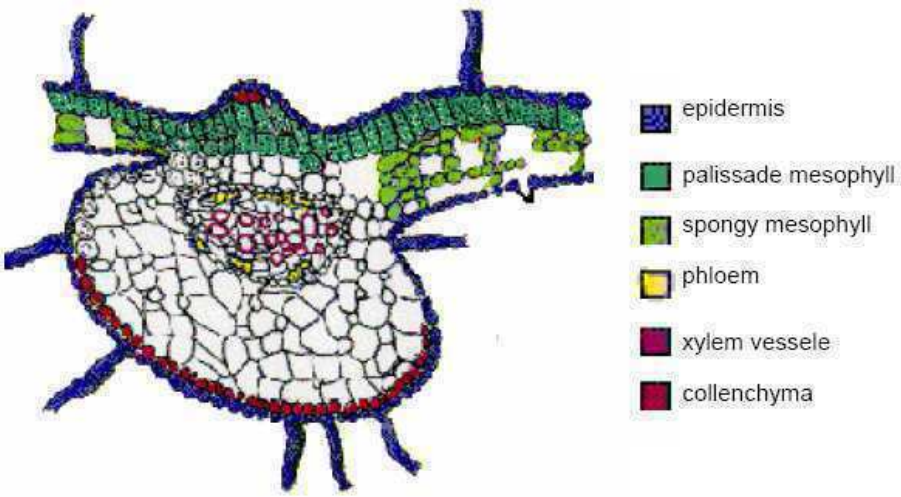

Figure 28: Cross section of a tomato leaf

Tomato flowers display both yellow fused anthers and pistil with generally six or seven petals/sepals. This flower structure allows autopollination.

Fruit development is divided in three stages: (1) a period of intensive cell division that begins at anthesis and continues for 2 weeks after fertilization; (2) a period of rapid cell expansion that begins toward the end of the cell division stage and continues until one week before the onset of ripening; (3) a ripening phase that initiates after growth has ceased and involves rapid chemical and structural changes that determine fruit aroma, color, texture and biochemical composition but not fruit size and shape (Figure 29).

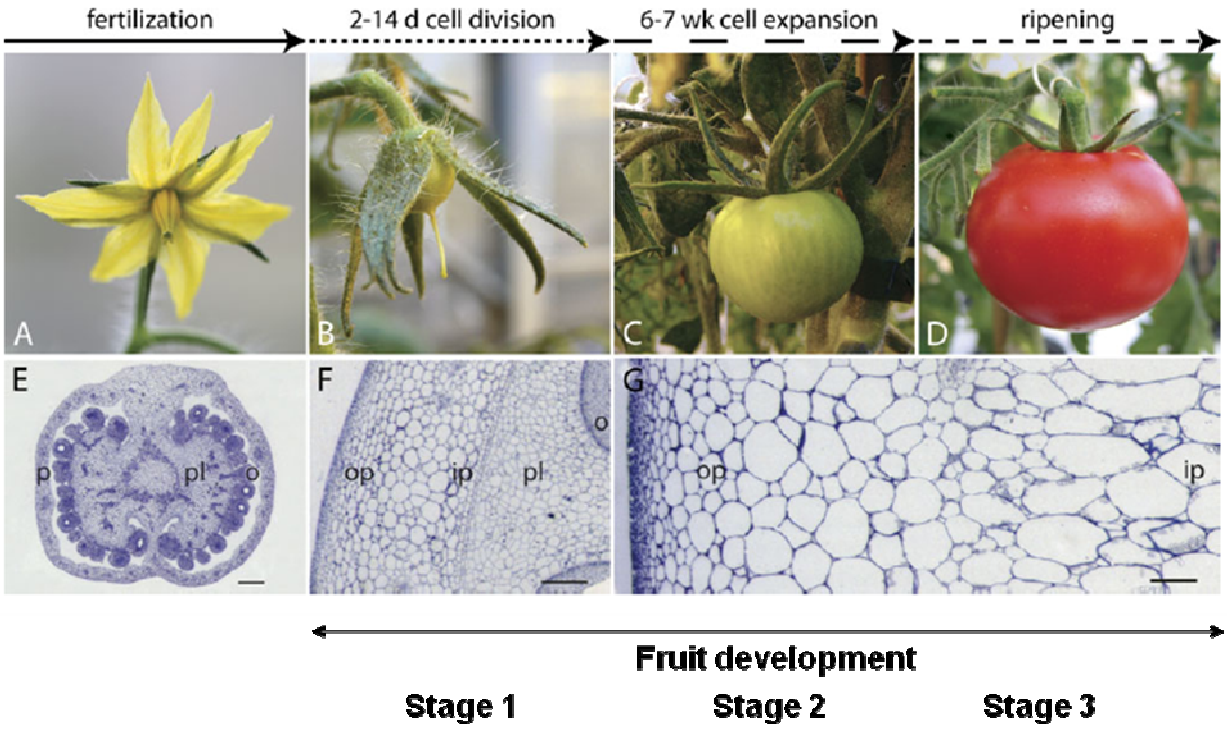

Figure 29: Overview of tomato fruit development. (A,E) Flower and micrograph of an ovary at anthesis, awaiting fertilization. (B,F) Fruit 10 days after fertilization and a micrograph of its pericarp. (C,G) Fruit 5 weeks after fertilization and a micrograph of its pericarp. (D) Ripe tomato fruit. P=pericarp, op=outer pericarp, ip=inner pericarp, pl=placenta, o=ovules. Bars=200µM. (de Jong et al. 2009).

The tomato fruit is a berry composed by an enpidermis, a thick pericarp surrounding the gynoecium constituted by columella and placental tissues around the seeds. The gynoecium is at least divided in two locules (Figure 29).

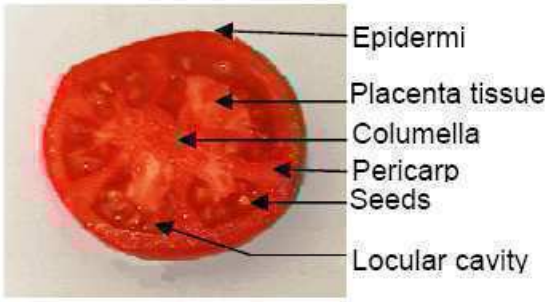

Figure 30: Cross section of tomato fruit

VII.4 The *MicroTom* cultivar

The main cultivar which was used during my PhD is the *MicroTom* cultivar. It was originally created for ornamental purposes (Scott and Harbaugh 1989). It resuls from the crossing of three cultivars and displays a very dwarf phenotype. *MicroTom* leaves are very small with deformed leaflets and present a deep green color compared to numerous cultivars (Figure 27). The MicroTom phenotype results from mutations in the *SELF-PRUNIG (SP)* and *DWARF (D)* genes. SP belongs to the CETS family of regulatory genes encoding modulator proteins that determine the potential for continuous growth of the shoot apical meristem (Pnueli et al. 2001). The *D* gene encodes a P450 protein involved in brassinosteroid (BR) biosynthesis (Bishop et al. 1999). Due to its small size, rapid growth and easy transformation it was chosen to generate numerous tomato transgenic lines (Meissner et al. 1997; Emmanuel and Levy 2002).

Chapter II: Analysis of Aux/IAA family in tomato

Résumé du chapitre II

Ce chapitre est consacré à l'identification et à la caractérisation de la famille multigénique des *Aux/IAA* chez la tomate. En effet, le grand nombre de protéines Aux/IAA pourrait expliquer en partie la diversité de réponse à l'auxine. Ce travail est présenté sour la forme d'une publication dans le journal Plant and Cell Physiology, intitulée « Genome-wide identification, functional analysis and expression profiling of the *Aux/IAA* gene family in tomato », dans laquelle je figure en deuxième auteur. Ma contribution concerne principalement en l'analyse de 7 des 25 gènes Aux/IAA, identifiés grâce au séquençage du génome de la tomate. Les Aux/IAA de tomate ont été nommés par comparaison phylogénétique avec les Aux/IAA d'Arabidopsis. Le nombre de gènes est légèrement diminué chez la tomate comparé à Arabidopsis (25 versus 29) en raison notamment d'une sous représentation des Aux/IAA dit non canoniques chez la tomate. La répartition chromosomique des Sl-IAA indique que ces gènes ont probablement évolués par duplication au sein du génome de la tomate. La première étape de l'étude fonctionnelle des gènes *Aux/IAA* a consisté en l'analyse de leurs profils d'expression spatio-temporelle. L'analyse par PCR quantitative du niveau d'expression des *Aux/IAA* de tomate dans différents organes de la plante montre qu'il est très variable selon le gène et le tissu considéré. Cette étude montre pour la première fois que la plupart des Aux/IAA ont leur expression régulée non seulement par l'auxine mais également par l'éthylène. Ce résultat laisse supposer qu'ils peuvent intervenir comme médiateur de la réponse à ces deux hormones. Par ailleurs, il a été montré que les Sl-IAA testés agissent en tant que répresseur de l'activité transcriptionnelle dépendante de l'auxine mais à des niveaux variables et qu'ils sont localisés au noyau. De manière intéressante, nous avons pu montrer que la protéine Sl-IAA32, qui ne possède pas le domaine de liaison au récepteur TIR1 de

l’auxine, n’est pas exclusivement localisée au noyau cellulaire mais est également présente dans le cytoplasme. Ce résultat, qui n’a jamais été décrit chez une autre espèce, indique que cette protéine pourrait de manière atypique avoir une fonction extranucléaire. Enfin, en complément des résultats présentés dans cette publication, une analyse structurelle de chacun des Sl-IAA a été réalisée.

I Introduction

The purpose of the Genomic and Fruits Biotechnology laboratory is to analyse how fruit development process is regulated notably by the phytohormones. Analyses of genes regulated during fruit initation, between flower bud stage and young fruit revealed that the genes most regulated during this process are related to auxin. Indeed 36% of total genes regulated between flower bud stage and flower at anthesis stage and 35% between flower at anthesis stage and the formation of young fruit (10 days after anthesis stage) are related to auxin (Wang.H et al. 2005). Moreover ethylene is the phytohorme known to drive fruit ripening in climacteric fruit but it is not able to trigger translation between fruit growth and fruit ripening. An increase in auxin concentration occurs at the end of fruit growth just before fruit ripening. Taken together these data suggested that auxin may be involved in regulating both fruit initiation and mechanism trigerring fruit ripening. To better undertand how auxin could regulate these processes the strategy chosen by the laboratory was to study the function of genes involved in auxin signaling using tomato plant as model. Indeed, as discussed in the previous chapter the tomato is a reference species for Solanacee and to study fleshy fruit development. To date, some knowledge is already available about how auxin metabolism, transport and signaling in tomato thanks to the characterization of genes involves in these processes.

As reported in previous chapter, auxin synthesis through IAOx pathway is mainly restricted to plant such as Arabidopsis and probably absent in tomato. This is supported by the absence of report of *CYP79*, *sur1* or *sur2* homologs. At the opposite, six *YUCCA* genes have been identified in tomato suggesting that IPA pathway is efficient in this specie (Expósito-

Rodríguez et al. 2011). In addition annotation of the tomato genome sequencing have identified two putative *TAA1* homologs (ITAG2.3: Solyc01g017610.1.1 and Solyc05g031600.1.1, http://solgenomics.net/). Regarding the auxin synthesis through IAM pathway, no report was made on *AMI1* in tomato but one putative homolog was identified (ITAG2.3: Solyc10g086170.1.1, http://solgenomics.net/). Nevertheless, no report and no homolog were identified for the *AAO* gene involved in TAM pathway. In conclusion, to date we can think that at least IPA and IAM auxin synthesis pathways are efficient in tomato plant. At polar auxin transport level, PIN and AUX/LAX auxin transporters have been identified in the tomato. Indeed, the tomato genome displays ten *PIN* (*SlPIN*1 to 10) and five *AUX/LAX* (*SlLAX*1 to 5) genes. Analysis of *SlPIN4* down-regulated plants notably revealed that these transgenic plants fail to develop normal secondary vascular tissues and secondary stems. This showed that like in Arabidopsis PIN proteins regulate tomato shoot architecture by controlling auxin transport. The analysis of *SlPIN* mutants revealed no fruit phenotype suggesting a functional redundancy of PIN proteins like in Arabidopsis (Krecek et al. 2009; Pattison and Catalá 2012). The pattern of expression of *PIN* and *AUX/LAX* genes in tomato is similar to thus in Arabidopsis. For example, both *SlPIN2* and *AtPIN2* show higher expression level in roots (Paponov et al. 2005; Pattison and Catalá 2012). Regarding more specifically fruit development, *PIN* and *AUX/LAX* genes are more expressed during fruit growth than ripening. Some of these genes display tissue-specific expression in fruits. Indeed, *SlPIN5* is preferentially expressed in seeds and fleshy tissue and *SlPIN7* and *SlLAX3* in the pericarp during the early stage of fruit growth (Pattison and Catalá 2012). To date no report has been made about PILS or ABCB transporters full families in tomato. Only one publication underlies the involvement of an ABC transporter in the regulation of tomato seed size, probably linked with a modification in auxin transport in this tissue (Orsi and Tanksley 2009).

Regarding auxin perception, ABP1 receptor is present in tomato (Christian et al. 2003). The TIR1 receptor has also been identified in tomato. The over-expression of *SlTIR1* results in dramatic phenotype with altered leaf formation, flower development and fruit initiation. It also leads to the formation of parthenocarpic fruit, underlying the importance of auxin perception for normal fruit development (Ren et al. 2011). To date no report has been made in tomato on AFB, the TIR1 homologs, or on the presence of a SKP2A receptor homolog. Nevertheless two putative SKP2A homologs have been annotated (ITAG2.3: Solyc06g049010.1.1 and Solyc10g076290.1.1, http://solgenomics.net/).

To understand how auxin regulates fruit development the laboratory focus on the study of genes involved in auxin signaling, the ARFs and Aux/IAAs genes. Thanks to tomato genome sequencing the 22 members of the ARF family were identified, and their pattern of expression was analyzed in different tissues and particularly during flower and fruit development (Kumar et al. 2011; Wu et al. 2011). At the beginning of my PhD 18 members of *Aux/IAA* family was identified and their pattern of expression was studied in different tomato tissues. Thanks to tomato genome sequencing seven other Aux/IAA genes were identified and analyzed. At the following is the article published in 2012 in the Plant Cell and Physiology Journal presenting results obtained regarding the characterization of *Sl-IAA* family, in which I am second author. I have notably studied the structure and expression of the seven *Aux/IAA* genes identified thanks to the genome sequencing (*Sl-IAA21*, *Sl-IAA22*, *Sl-IAA23*, *Sl-IAA32*, *Sl-IAA33, Sl-IAA35* and *Sl-IAA36*) and analyzed the subcellular localization of Aux/IAA proteins. Moreover, I have also performed statistical analysis of both expression and cytometer data.

II Publication

PCP
PLANT & CELL PHYSIOLOGY

Genome-Wide Identification, Functional Analysis and Expression Profiling of the *Aux/IAA* Gene Family in Tomato

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Regular Paper

Auxin is a central hormone that exerts pleiotropic effects on plant growth including the development of roots, shoots, flowers and fruit. The perception and signaling of the plant hormone auxin rely on the cooperative action of several components, among which auxin/indole-3-acetic acid (Aux/IAA) proteins play a pivotal role. In this study, we identified and comprehensively analyzed the entire *Aux/IAA* gene family in tomato (*Solanum lycopersicum*), a reference species for Solanaceae plants, and the model plant for fleshy fruit development. Functional characterization using a dedicated single cell system revealed that tomato Aux/IAA proteins function as active repressors of auxin-dependent gene transcription, with, however, different Aux/IAA members displaying varying levels of repression. Phylogenetic analysis indicated that the *Aux/IAA* gene family is slightly contracted in tomato compared with Arabidopsis, with a lower representation of non-canonical proteins. *Sl-IAA* genes display distinctive expression pattern in different tomato organs and tissues, and some of them display differential responses to auxin and ethylene, suggesting that Aux/IAAs may play a role in linking both hormone signaling pathways. The data presented here shed more light on *Sl-IAA* genes and provides new leads towards the elucidation of their function during plant development and in mediating hormone cross-talk.

Keywords: Auxin • Aux/IAA • Ethylene • Expression analysis • Tomato • Transcriptional repressor.

Abbreviations: AFB, auxin receptor F-box; ARF, auxin response factor; Aux/IAA, auxin/indole-3-acetic acid; AuxRE, auxin-responsive *cis*-element; CaMV, *Cauliflower mosaic virus*; EAR, ethylene-responsive element-binding factor-associated amphiphilic repression; EST, expressed sequence tag; GFP, green fluorescent protein; MS medium, Murashige and Skoog medium; NLS, nuclear localization signal; qRT–PCR, quantitative reverse transcription–PCR; *SAUR*, small auxin up RNA; SGN, Solanaceae Genomics Network; Sl-IAA, *Solanum lycopersicum* auxin/indole-3-acetic acid; TIR1, transport inhibitor response1; TPL, topless; YFP, yellow fluorescent protein.

The nucleotide sequence data from this article can be found in the Genbank/EMBL data libraries under the following accession numbers: JN379431 (*Sl-IAA1*), JN379432 (*Sl-IAA2*), JN379433 (*Sl-IAA3*), JN379434 (*Sl-IAA4*), JN379435 (*Sl-IAA7*), JN379436 (*Sl-IAA8*), JN379437 (*Sl-IAA9*), JN379438 (*Sl-IAA11*), JN379439 (*Sl-IAA12*), JN379440 (*Sl-IAA13*), JN379441 (*Sl-IAA14*), JN379442 (*Sl-IAA15*), JN379443 (*Sl-IAA16*), JN379444 (*Sl-IAA17*), JN379445 (*Sl-IAA19*), JN379446 (*Sl-IAA21*), JN379447 (*Sl-IAA22*), JN379448 (*Sl-IAA23*), JN379449 (*Sl-IAA26*), JN379450 (*Sl-IAA27*), JN379451 (*Sl-IAA29*), JN379452 (*Sl-IAA32*), JN379453 (*Sl-IAA33*), JN379454 (*Sl-IAA35*), JN379455 (*Sl-IAA36*).

Introduction

The perception and signaling of the plant hormone auxin involve the cooperative action of several components, among which auxin/indole-3-acetic acid (Aux/IAA) proteins play a pivotal role. Aux/IAA proteins were shown to be a direct target of the auxin transport inhibitor response1 (TIR1) and of its paralogs AUXIN RECEPTOR F-BOX/AFB1 and AFB3F-box receptors (AFBs) (Dharmasiri et al. 2005a, Dharmasiri et al. 2005b, Kepinski and Leyser 2005, Tan et al. 2007). Binding of auxin to its receptors leads to the degradation of Aux/IAA proteins. This auxin-dependent proteolysis releases auxin response factors (ARFs) that otherwise remain trapped via their binding to Aux/IAA partners. The *Aux/IAA* genes represent a class of primary auxin-responsive genes which were shown to be, in the majority, rapidly induced by auxin (Theologis et al. 1985, Oeller et al. 1993, Yamamoto and Yamamoto 1998). Aux/IAAs are described as short-lived and nuclear-localized proteins (Hagen and Guilfoyle 2002, Liscum and Reed 2002), and biochemical and genetic studies indicated that they generally function as transcriptional repressors of auxin-regulated genes (Tiwari et al. 2001, Tiwari et al. 2004). Canonical Aux/IAA

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proteins share four conserved amino acid sequence motifs known as domains I, II, III and IV, although several proteins lacking one or more of these domains are also included in the family (Reed 2001). Domain I is a repressor domain that contains a conserved leucine repeat motif (LxLxLx) similar to the so-called EAR (ethylene-responsive element-binding factor-associated amphiphilic repression) domain (Tiwari et al. 2004). Domain I is also required for the recruitment of the transcriptional co-repressor TOPLESS (Szemenyei et al. 2008). Domain II confers protein instability, leading to rapid degradation of Aux/IAA through the interaction with the F-box protein TIR1 (a component of the SCF^{TIR1} ubiquitin ligase complex) (Dharmasiri et al. 2005a, Dharmasiri et al. 2005b, Kepinski and Leyser 2005, Tan et al. 2007). In fact, mutations in Aux/IAA domain II resulted in increased protein accumulation leading to auxin-related developmental phenotypes (Reed 2001, Liscum and Reed 2002, Uehara et al. 2008). The C-terminal domains III and IV are shared with ARF proteins, and are known to promote homo- and heterodimerization of Aux/IAA polypeptides, as well as interaction between Aux/IAAs and ARFs (Remington et al. 2004, Overvoorde et al. 2005). Aux/IAAs impact the transcriptional activity of target genes through the binding to their ARF partners. ARF proteins are capable of binding to the auxin-responsive *cis*-element (AuxRE) present upstream of the coding sequence of auxin-responsive genes (Ulmasov et al. 1997). Depending on the amino acid composition of their variable internal region, the ARF proteins can either activate or repress gene transcription (Ulmasov et al. 1999). Most of our understanding of the diverse roles of Aux/IAAs in planta is based on the characterization of gain-of-function mutants in the Arabidopsis model plant, whereas phenotypes associated with loss of function are scarce probably due to important functional redundancy among Aux/IAA family members (Overvoorde et al. 2005). In contrast, down-regulation of various *Aux/IAA* genes in the Solanaceae species results in visible and distinct phenotypes. Down-regulation of the tomato (*Solanum lycopersicum*) *Sl-IAA9* resulted in pleiotropic phenotypes, consistent with its ubiquitous expression pattern (Wang et al. 2005). *Sl-IAA9*-inhibited lines also displayed some specific phenotypes such as entire leaves and parthenocarpic fruit, indicating that Sl-IAA9 is a key regulator of fruit set and leaf morphogenesis (Wang et al. 2005, Wang et al. 2009). Down-regulation of another *Aux/IAA* gene in tomato, *Sl-IAA3*, results in both auxin- and ethylene-associated phenotypes including altered apical dominance, lower auxin sensitivity, exaggerated apical hook curvature in the dark and reduced petiole epinasty in the light, thus revealing new roles for *Aux/IAA* genes (Chaabouni et al. 2009a). These data position Sl-IAA3 at the crossroads of auxin and ethylene signaling in tomato (Chaabouni et al. 2009b). More recently, it was shown that Sl-IAA15 is involved in trichome development as *Sl-IAA15*-down-regulated lines display strong reduction of type I, V and VI trichomes (Deng et al. 2012). Likewise, suppression of St-IAA2 in *Solanum tuberosum* results in clear phenotypes including increased plant height, petiole hyponasty and curvature of growing leaf primordia in the shoot apex (Kloosterman et al. 2006). These data do not support the functional redundancy among *Aux/IAA* genes generally described in the plant model Arabidopsis and clearly emphasize the need to widen the functional characterization to other plant species in order to decipher thoroughly the physiological significance of different Aux/IAA family members. To lay the foundation for a better understanding of the Aux/IAA family in the Solanaceae family, the present study identified and comprehensively analysed the entire *Aux/IAA* gene family in tomato (*S. lycopersicum*), a reference species for Solanaceae plants. Phylogenetic analysis revealed that some Aux/IAA clades are either expanded or retracted in tomato compared with Arabidopsis. Expression studies revealed a distinctive spatio-temporal pattern of expression for tomato *Aux/IAA* genes, some of which display differential responsiveness to auxin and ethylene.

Results

Identification and sequence analysis of the tomato *Sl-IAA* gene family members

Aux/IAA genes belong to a large gene family found in all plant species ranging from 26 members in *Sorghum bicolor* (S. Wang et al. 2010) to 35 in poplar (Kalluri et al. 2007). In Arabidopsis, this gene family comprises 29 members (Liscum and Reed 2002) while it contains 31 in rice and maize (Jain et al. 2006, Y. Wang et al. 2010). To shed more light on this gene family, structural and functional characterizations of the tomato *Aux/IAA* genes were carried out. Both BLASTN and TBLASTN search were performed on the whole set of tomato unigenes in the SGN database (Solanaceae Genomics Network, http://www.sgn.cornell.edu/) using either partial tomato *Aux/IAA* clones (Nebenführ et al. 2000, Jones et al. 2002) or Aux/IAA Arabidopsis protein sequences. This search was further extended taking advantage of the recent sequence information generated by the tomato genome sequencing project (Solanaceae Genomics Network, http://www.sgn.cornell.edu/). In addition, the predicted proteome deduced from the tomato genome was searched against the pfam AUX_IAA hidden-Markov model (PF02309) recognizing both AUX-IAA and ARF protein sequences (Finn et al. 2010) using the HMMER3 software. This HMM-based search identified 24 *Aux//IAA* genes in the tomato genome annotation (ITAG Release 2.3 predicted CDS). With the exception of *Sl-IAA21*, all the *Aux/IAA* genes identified in this work are present in the tomato genome annotation file iTAG2.30. Overall, this in silico search resulted in the identification of 25 tomato genes displaying the conserved features of *Aux/IAA* (**Supplementary Table S1**). The coding sequences of these genes were submitted to GenBank/EMBL. The size of the deduced Aux/IAA proteins varies greatly, ranging from 147 amino acids (Sl-IAA33) to 349 amino acids (Sl-IAA9), and the corresponding molecular mass varies from 16 to 37 kDa (**Supplementary Table S2**). The predicted isoelectric point

also varies widely from 5.02 (Sl-IAA32) to 9.08 (Sl-IAA15) (**Supplementary Table S2**), suggesting that different Aux/IAA proteins might operate in different microenvironments. Pair-wise comparisons of these Sl-IAA protein sequences showed that the identity level ranges from as low as 19% (between Sl-IAA33 and Sl-IAA8/Sl-IAA27) to a highly identical level of 79% (Sl-IAA21 and Sl-IAA23) (**Supplementary Table S3**). The overall identity among the various proteins is low, even between members of the same phylogenetic branch (**Supplementary Fig. S1**). Alignment of amino acid sequences of tomato and Arabidopsis Aux/IAAs revealed the typical four highly conserved domains found in canonical Aux/IAA proteins (Reed 2001), with the exception of Sl-IAA32 which lacks domain II and Sl-IAA33 missing domains I and II and containing only a weakly conserved domain III (**Fig. 1**). Therefore, Sl-IAA32 and Sl-IAA33 can be considered as non-canonical Aux/IAA proteins like their putative orthologs in Arabidopsis (Dreher et al. 2006).

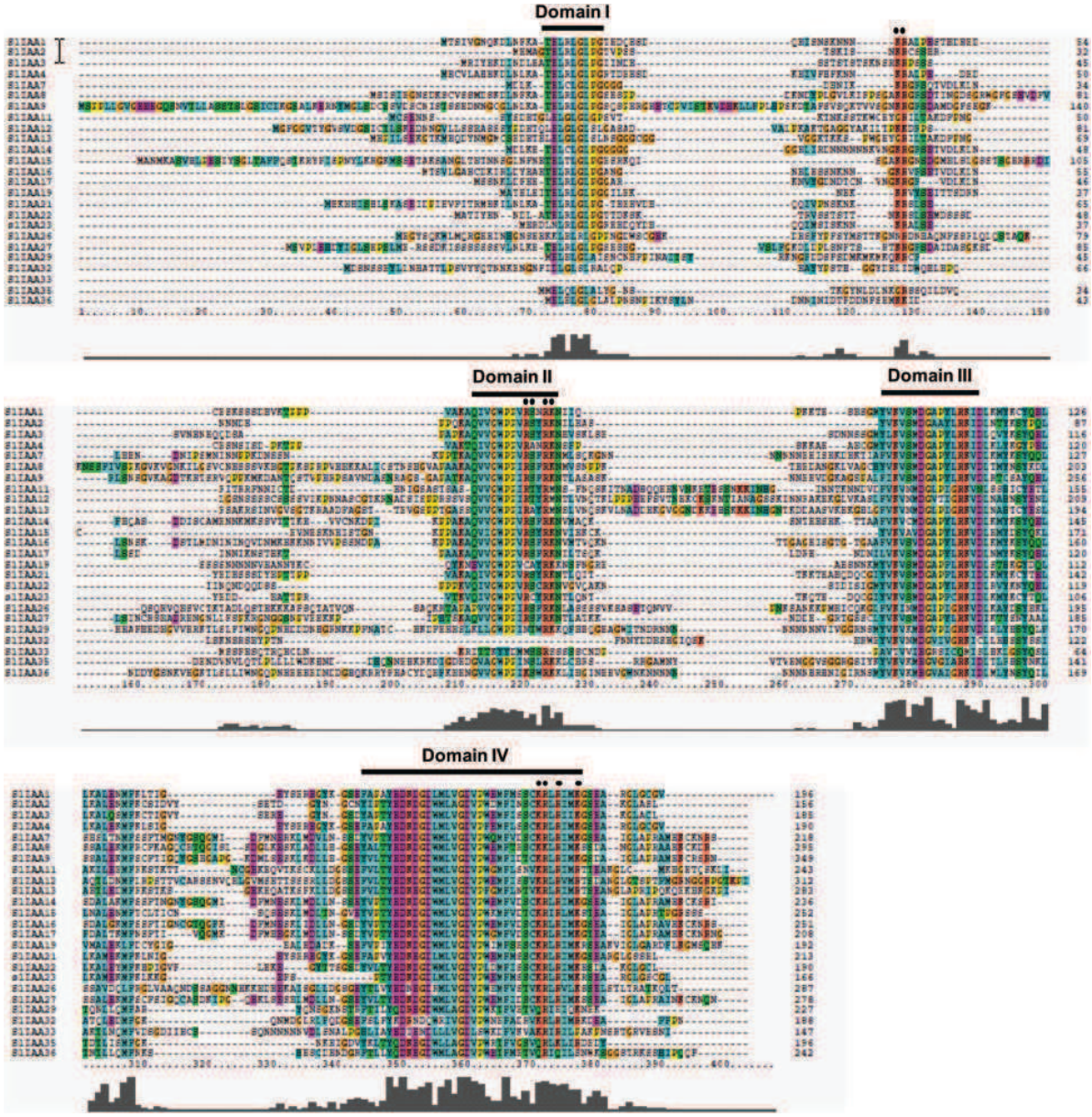

Fig. 1 Multiple sequence alignment of the full-length Sl-IAA proteins obtained with ClustalX and manual correction. Conserved domains of Aux/IAA proteins are underlined. Nuclear localization signals (NLSs) are indicated by filled circles. The amino acid position is given on the right of each sequence.

Phylogenetic analysis of Aux/IAAs

Phylogenetic analysis was conducted to assess the relationship between tomato and Arabidopsis Aux/IAAs. The tomato *Aux/IAA* genes were renamed to comply with the nomenclature of their closest Arabidopsis homologs. **Supplementary Fig. S1** shows that Aux/IAA proteins group into 11 distinct clades named here A–K. Overall, the tomato family is slightly contracted (25 members) compared with the size of that of Arabidopsis (29 members). With reference to Arabidopsis, four clades (D, F, G and I) are contracted in the tomato and two (A and J) are expanded. Clade A includes seven genes in tomato but only four members in Arabidopsis, while clade J is comprised of three genes in tomato and contains a single member in Arabidopsis. The non-canonical clade H lacking the conserved domains II contains three members (AtIAA20, AtIAA30 and AtIAA31) in Arabidopsis but is not represented in tomato. Clade I, which also gathers non-canonical Aux/IAAs lacking either one or two of the conserved domains, is represented by two Aux/IAAs in Arabidopsis (AtIAA32 and AtIAA34) but only by a single member in tomato (Sl-IAA32). Overall, the non-canonical Aux/IAAs are over-represented in Arabidopsis with six genes (*AtIAA20*, *AtIAA30*, *AtIAA31*, *AtIAA32*, *AtIAA33* and *AtIAA34*), while only two were found in tomato (*Sl-IAA32* and *Sl-IAA33*).

Chromosomal distribution of *Sl-IAA* genes

The *Sl-IAA* sequences were initially mapped on the tomato genome using the introgression line population obtained by crossing and successive back-crossing of cultivated *S. lycopersicum* with *Solanum pennelli* (Eshed and Zamir 1995), and the mapping was subsequently refined using the SGN Tomato Whole Genome Scaffolds data (2.40) (http://www.sgn.cornell.edu/tools/blast/; The International Tomato Genome Sequencing Consortium). The 25 tomato *Aux/IAA* genes are distributed among nine tomato chromosomes (**Supplementary Fig. S2**), with chromosomes 2, 10 and 11 being devoid of *Aux/IAA* genes. Six *Sl-IAA* genes are present on chromosome 6; five on chromosomes 3 and 9; two on chromosomes 4, 7 and 12; and one on chromosomes 1, 5 and 8. The *Aux/IAA* genes tend to be clustered in preferential genomic regions, with the presence of closely adjacent genes on chromosome 3 (*Sl-IAA19*, *Sl-IAA15*, *Sl-IAA27* and *Sl-IAA26*), chromosome 6 (*Sl-IAA22*, *Sl-IAA17* and *Sl-IAA7*, *Sl-IAA4*) and chromosome 9 (*Sl-IAA1* and *Sl-IAA14*). Remarkably, the four contiguous tomato *Aux/IAA* genes mapped on chromosome 3 are located in a region spanning <0.5 Mb. On chromosome 6, *Sl-IAA22* and *Sl-IAA17* display an inverted orientation and are separated by only 7.5 kb. Likewise, in another locus of chromosome 6, *Sl-IAA7* and *Sl-IAA4* show a similar situation, being 23.6 kb apart. The same situation prevails in chromosome 9 where *Sl-IAA1* and *Sl-IAA14* are 32 kb apart. These data suggest that the distribution of some *Sl-IAA* genes on the tomato genome probably results from either reverse or direct tandem duplication.

Aux/IAA proteins are nuclear localized

Two types of putative nuclear localization signals (NLSs) were detected in most of the Aux/IAA proteins. Generally, tomato Aux/IAA proteins display two conserved nuclear localization domains: (i) a bipartite structure comprising a conserved KR basic doublet between domains I and II associated with the presence of basic amino acids in domain II; and (ii) a basic residue-rich region located in domain IV that resembles the SV40-type NLS (**Fig. 1**). However, some Sl-IAAs display imperfect or weakly conserved nuclear targeting motifs. For instance, Sl-IAA35 lacks the two conserved NLSs, while Sl-IAA32 and Sl-IAA33 lack the bipartite structure and Sl-IAA29 and Sl-IAA36 contain a degenerated NLS. The ability of the degenerated NLS present in Sl-IAA29 and the absence of the bipartite structure in Sl-IAA32 to target the protein to the nucleus was assessed by transient expression assay. To this end, the coding sequence of the selected *Aux/IAA* genes was fused in-frame to either GFP (green fluorescent protein) or YFP (yellow fluorescent protein) coding sequences and expressed under the control of the 35S promoter of *Cauliflower mosaic virus* (CaMV) in tobacco protoplasts. Two Aux/IAAs with a conserved NLS (Sl-IAA4 and Sl-IAA22) were used as reference proteins for nuclear targeting. Fluorescence microscopy analysis demonstrated that in contrast to control cells transformed with GFP alone where the fluorescence was found throughout the cell, the Sl-IAA4–GFP and Sl-IAA22–YFP fusion proteins were exclusively localized to the nucleus (**Fig. 2**). Likewise, Sl-IAA29–YFP was also strictly targeted to the nucleus, suggesting that the degenerated NLS was sufficient to drive the protein specifically to the nucleus. In contrast, though Sl-IAA32–YFP was localized in the nucleus, the accumulation of the protein was not restricted to this compartment (**Fig. 2**). The extension of the Sl-IAA32 localization to the extranuclear compartment was probably due to the lack of the bipartite NLS and/or the absence of domain II responsible for protein degradation. Taken together, the nuclear targeting of the tomato Aux/IAA proteins is consistent with a putative transcriptional regulatory function.

Tomato Aux/IAA proteins function as active repressors of auxin-dependent transcription

The ability of the tomato Aux/IAA proteins to regulate in vivo the activity of the synthetic DR5 auxin-responsive promoter fused to the GFP reporter gene (Ottenschlager et al. 2003) was investigated by transient expression experiments using tobacco BY-2 protoplasts. The DR5::GFP reporter construct was used to assess auxin-dependent transcriptional activity based on the presence in the DR5 promoter of several copies of the TGTCTC core motif that makes up the AuxRE (Ulmasov et al. 1997). In our system, DR5-driven GFP expression was enhanced up to 10-fold by auxin treatment, and co-transfection of the reporter construct with a mock effector plasmid containing the 35S promoter but lacking Sl-IAA coding sequence did not impact the auxin induction of the DR5 activity (**Fig. 3A**). While all Sl-IAA proteins were able to

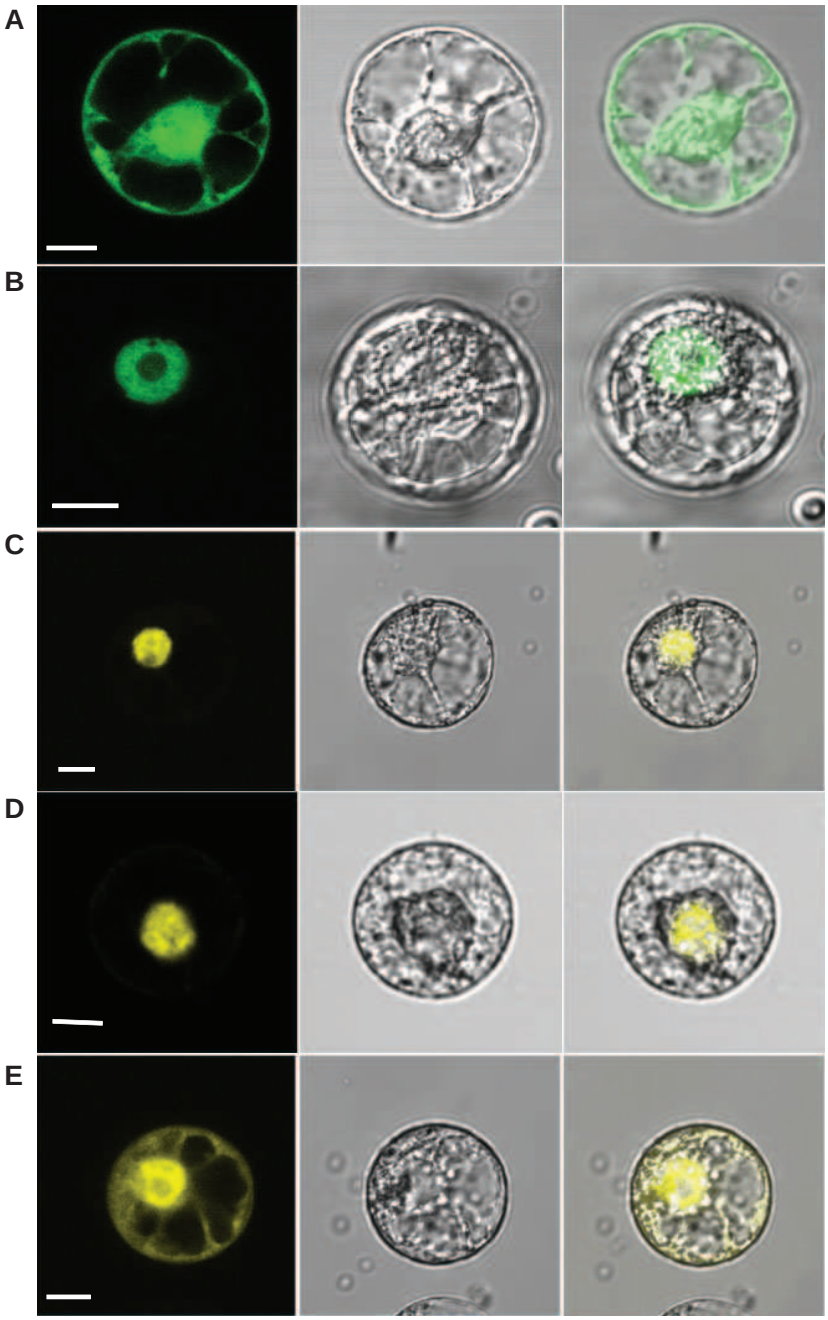

Fig. 2 Subcellular localization of Sl-IAA4, Sl-IAA22, Sl-IAA29 and Sl-IAA32 proteins. Sl-IAA4–GFP, Sl-IAA22–YFP, Sl-IAA29–YFP and Sl-IAA32–YFP fusion proteins were transiently expressed in BY-2 tobacco protoplasts, and their subcellular localization was analyzed by confocal laser scanning microscopy. The merged pictures of the green or yellow fluorescence channel (left panels) and the corresponding bright field (middle panels) are shown (right panels). (A) Control cells expressing GFP alone. (B) Cells expressing the Sl-IAA4–GFP fusion protein. (C) Cells expressing the Sl-IAA22–YFP fusion protein. (D) Cells expressing the Sl-IAA29–YFP fusion protein. (E) Cells expressing the Sl-IAA32–YFP fusion protein. The scale bar indicates 10 μm.

repress the auxin-induced expression of the DR5 promoter, the repression levels ranged from 23 to 87% (**Fig. 3A**), indicating that some proteins are strong repressors, e.g. Sl-IAA8, Sl-IAA9, Sl-IAA13 and Sl-IAA26, while others, e.g. Sl-IAA1, Sl-IAA11, Sl-IAA12 and Sl-IAA19, are weak repressors. The repression activity of Aux/IAA proteins is consistent with the presence of an LxLxL repression motif in domain I in all tomato Sl-IAA proteins tested, a motif that was shown to be important in

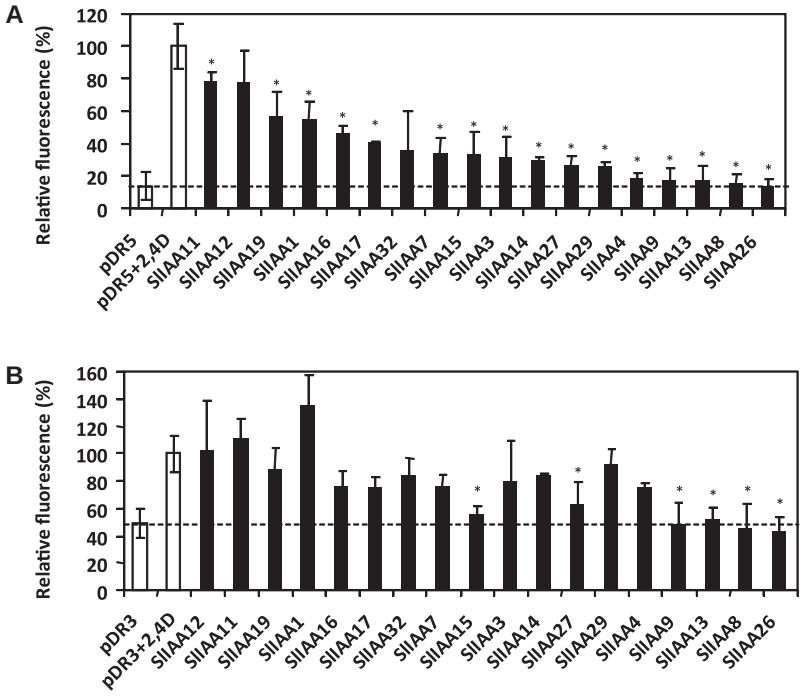

Fig. 3 Repressor activity of Aux/IAA proteins on a synthetic promoter and the native *Sl-IAA3* promoter. Transient expression in a single cell system has been used to assess the repression activity of Aux/IAA proteins on auxin-induced transcription of the GFP reporter gene driven by auxin-responsive promoters. The fluorescence of the reporter gene was measured by flow cytometry upon treatement with 50 μM 2,4-D and co-transfection with a reporter construct (DR5::GFP or Sl-IAA3promoter::GFP) and an effector construct (35S::SlIAA). The basal fluorescence obtained in the mock assay transfected with the reporter construct and an empty effector construct in the presence of auxin treatment was taken as reference (100% relative fluorescence). Biological triplicates were averaged and analyzed statistically using a Student *t*-test (*$P < 0.05$). Bars indicate the SEM. (A) Aux/IAA activity on a synthetic DR5 promoter gene containing nine TGTCTC boxes. (B) Aux/IAA activity on the auxin-inducible native *Sl-IAA3* promoter containing two TGTCCC boxes.

conferring repression activity in Arabidopsis Aux/IAAs (**Table 1**; Tiwari et al. 2004). No correlation was found between the level of repression and the amino acid environment surrounding the LxLxL motif present in domain I (**Fig. 3A**, **Table 1**). Among all the tomato Aux/IAA proteins, 12 contain the more representative domain I (TE**L**R**L**G**L**PG); however, these proteins displayed different levels of repression. For instance, Sl-IAA8 totally repressed the auxin-induced DR5 activity whereas Sl-IAA19 repressed only 50% of this activity. Moreover, Sl-IAA26 and Sl-IAA8 which contain the kk**L**e**L**r**L**gp and TE**L**R**L**G**L**PG type of domain I, respectively, were both capable of completely repressing DR5 activity (**Fig. 3A**, **Table 1**). Neither the length nor the number of repeats of this motif correlate with the level of transcriptional repression displayed by the tomato Aux/IAAs. Indeed, Sl-IAA12 has an expanded repression motif made up of five leucine repeats but only displayed a weak repression activity (**Fig. 3A**; **Supplementary Fig. S3A**). Likewise, the presence of two conserved repression motifs (LxLxLx and DLxLxL) in Sl-IAA16, Sl-IAA17 and Sl-IAA7 proteins did not result in stronger repression activity (**Fig. 3A**; **Supplementary Fig. S3B**). Overall, these results are consistent with tomato Aux/IAA proteins being transcriptional repressors on TGTCTC-containing promoters.

The repressor activity of the tomato Aux/IAAs was also tested with a native tomato auxin-responsive promoter, the *Sl-IAA3* promoter carrying degenerated AuxREs (TGTCCC). Among all the tomato Aux/IAAs tested, only six Sl-IAAs (Sl-IAA8, Sl-IAA9, Sl-IAA13, Sl-IAA15, Sl-IAA26 and Sl-IAA27) showed significant repression activity on the native *Sl-IAA3* promoter (**Fig. 3B**). All these repressors were even able to abolish totally the auxin-induced expression of the *Sl-IAA3* promoter-driven GFP (**Fig. 3B**). The remaining Aux/IAA proteins displayed no, or only partial, repression activity on the native auxin-responsive promoter. Moreover, the Aux/IAAs showing the strongest repression activity (Sl-IAA8, Sl-IAA9, Sl-IAA13 and Sl-IAA26) on the synthetic DR5 promoter were also those displaying the highest repression on the native *Sl-IAA3* promoter. Likewise, the Aux/IAAs showing weak repression activity on the synthetic promoter also displayed no repression on the native *Sl-IAA3* promoter (Sl-IAA1, Sl-IAA11, Sl-IAA12 and Sl-IAA19). The slight differences observed between the synthetic DR5 and the native *Sl-IAA3* promoter are likely to be due to the complexity of the latter promoter which contains several *cis*-regulatory elements, independent of auxin regulation (Chaabouni et al. 2009a).

Table 1 LxLxLx motifs in tomato Aux/IAA repression domain I

Protein name	Domain I
Sl-IAA1	TE**L**R**L**G**L**PG
Sl-IAA2	TE**L**R**L**G**L**PG
Sl-IAA3	TE**L**R**L**G**L**PG
Sl-IAA4	TE**L**R**L**G**L**PG
Sl-IAA9	TE**L**R**L**G**L**PG
Sl-IAA15	TE**L**R**L**G**L**PG
Sl-IAA16	TE**L**R**L**G**L**PG
Sl-IAA17	TE**L**R**L**G**L**PG
Sl-IAA19	TE**L**R**L**G**L**PG
Sl-IAA21	TE**L**R**L**G**L**PG
Sl-IAA22	TE**L**R**L**G**L**PG
Sl-IAA8	TE**L**R**L**G**L**PG
Sl-IAA14	TE**L**c**L**G**L**PG
Sl-IAA7	TE**L**c**L**G**L**PG
Sl-IAA27	TE**L**t**L**G**L**PG
Sl-IAA13	TE**L**e**L**G**L**gl
Sl-IAA23	In**L**R**L**G**L**PG
Sl-IAA11	Tg**L**e**L**G**L**gl
Sl-IAA12	Tq**L**e**L**G**L**gl
Sl-IAA29	mE**L**e**L**G**L**ai
Sl-IAA36	mE**L**e**L**G**L**gl
Sl-IAA35	mE**L**q**L**G**L**al
Sl-IAA26	kk**L**e**L**r**L**gp
Sl-IAA32	id**L**g**L**s**L**ra

Conserved leucine residues in the LxLxLx motif are in bold. The most conserved amino acids in domain I of *A. thaliana* (Tiwari et al. 2004) and tomato Aux/IAA proteins are in uppercase. Sl-IAA33 which lacks a LxLxL motif is not shown.

Expression analysis of tomato Aux/IAA genes

Full-length cDNAs were amplified for 22 *Aux/IAA* genes attesting to their expression at least at the transcriptional level in different tomato plant tissues and organs. For the remaining three tomato *Aux/IAA* genes (*Sl-IAA21*, *Sl-IAA23* and *Sl-IAA33*), no corresponding cDNA could be isolated from the various plant tissues tested. In addition, with the exception of *Sl-IAA33* for which an expressed sequence tag (EST) was available from suspension cell culture, no sequence was identified for these genes in the available EST databases (Tomato gene index project: http://compbio.dfci.harvard.edu/tgi/cgi bin/tgi/gimain.pl?gudb=tomato; SGN: http://www.sgn.cornell.edu; KaFTom: http://www.pgb.kazusa.or.jp/kaftom/ ; MiBASE http://www.kazusa.or.jp/jsol/microtom/indexe.html). This supports the idea that these latter genes might be either preferentially expressed in small subsets of cells or not expressed at all.

To gain insight into the spatial pattern of expression of *Sl-IAA* genes, their transcript accumulation was assessed in different plant tissues and organs. The expression pattern was studied by quantitative reverse transcription–PCR (qRT–PCR) for 19 out of the 22 expressed *Aux/IAA* genes. The Treeview presented in **Fig. 4** gathers the qRT–PCR data of 19 *Sl-IAA* genes using RNA samples corresponding to seven different plant tissues. The clustering revealed four main clades. *Aux/IAA* genes from clade 1 correspond to family members displaying the highest expression in fruit tissues. In contrast, genes in clade 2 and 3 displayed higher expression in vegetative tissues while clade 4 corresponded to *Aux/IAA* genes with a low level of expression in all tissues. No correlation was found between the clustering based on the expression pattern and that generated based on phylogenetic analysis (**Supplementary Fig. S1**; **Fig. 4**). For most *Aux/IAA* genes the highest expression level was found in young leaves and seedlings, two tissues where auxin is known to play an important role. Some *Aux/IAA* genes displayed clear preferential expression in a specific tissue, such as *Sl-IAA15* showing the highest expression in mature green fruit, *Sl-IAA7* and *Sl-IAA19* in young leaves and *Sl-IAA26* and *Sl-IAA29* in seedlings (**Fig. 5**). The expression of *Sl-IAA9*, *Sl-IAA13* and *Sl-IAA27* showed minimal variation between tissues, suggesting that the regulation of these genes might take place essentially at the post-translational level. Overall, the tissue-preferential expression displayed by some *Aux/IAA* genes could be indicative of their involvement in specific plant tissues and developmental processes.

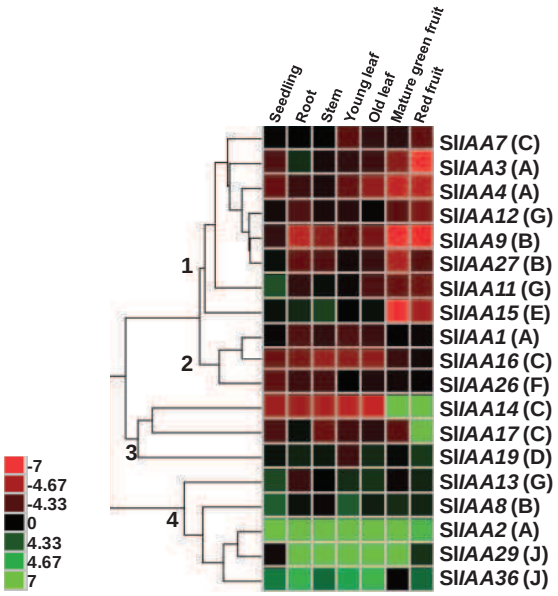

Fig. 4 Heatmap showing the expression of *Sl-IAA* genes in different tissues. Quantitative RT–PCR was used to assess *Sl-IAA* transcript accumulation in total RNA samples extracted from seedling, roots, stem, young leaf, old leaf, mature green fruit and red fruit. Values represent the best experiment among three independent biological replicates. Genes highly or weakly expressed in the tissues are colored red and green, respectively. The heat map was generated using cluster 3.0 software. The number in parentheses designates the phylogenetic clade of each *Aux/IAA* gene.

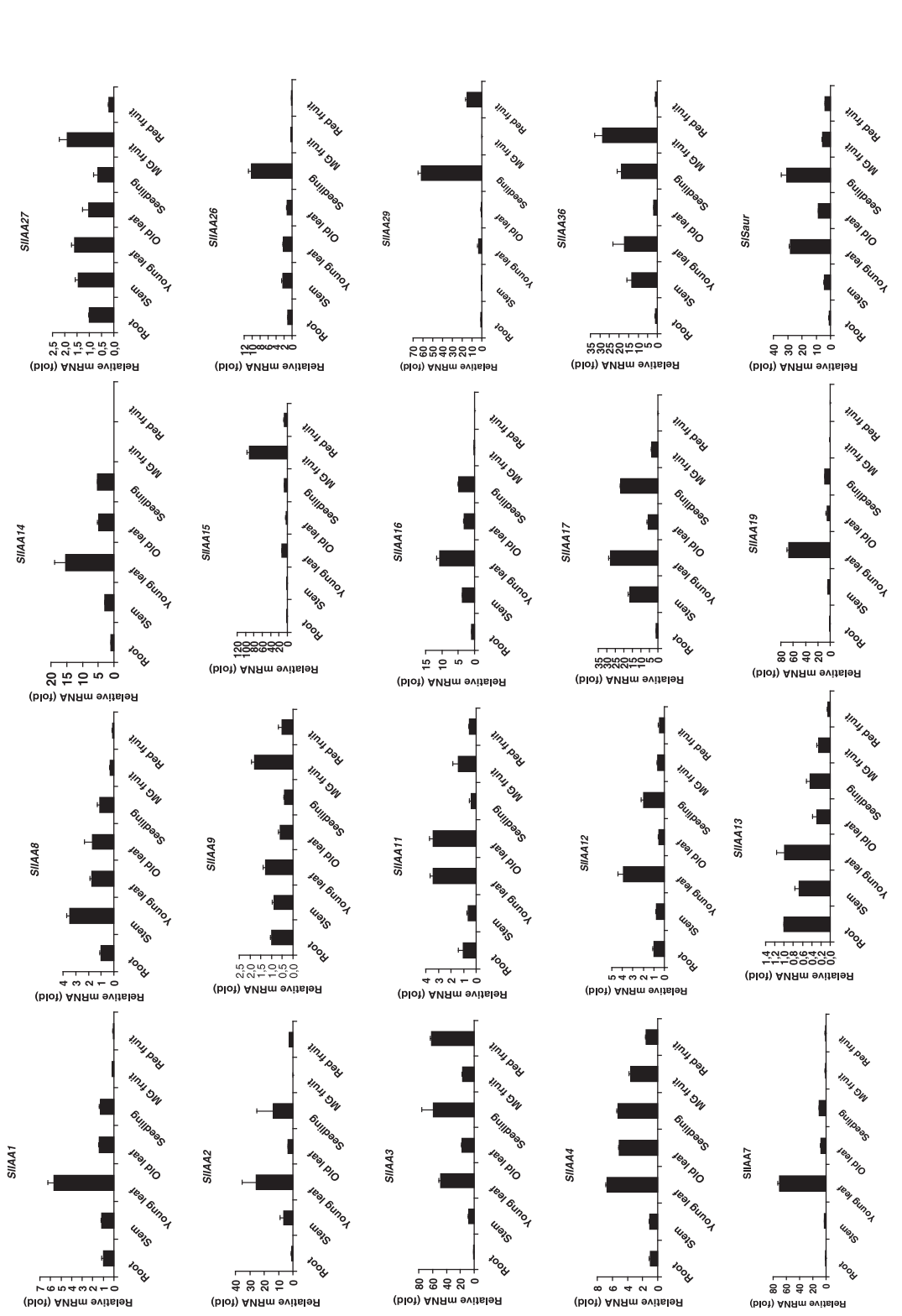

Fig. 5 Real-time PCR expression profiles of individual *Sl-IAA* genes. The relative mRNA level of individual *Sl-IAA* genes was normalized with respect to the housekeeping gene, actin, in different tissues. The results were expressed using the root as a reference for each gene (relative mRNA level 1). Values represent the best experiment among three independent biological repetitions. Bars indicate the SEM of three experimental repetitions.

Auxin and ethylene responsiveness of tomato *Aux/IAA* genes

The first *Aux/IAA* genes were isolated from various plant species based on their rapid induction in response to auxin. Screening for the presence of *cis*-acting elements within promoter regions (2 kb from the start codon) using the Place database (http://www.dna.affrc.go.jp/PLACE/signalscan.html) revealed that the majority of the *Sl-IAA* promoters contain AuxREs as either a conserved (TGTCTC) or degenerate (TGT CCC) motif. In addition to the AuxREs, 16 out of the 25 *Sl-IAA* promoters contain conserved ethylene-response motifs, the so-called ERELEE4 motif found in the tomato E4 gene (AWTT CAAA) (**Supplementary Table S1**). The presence of these *cis*-regulatory elements suggests a potential regulation of the *Aux/IAA* genes by both auxin and ethylene. The ethylene and auxin responsiveness of the *Sl-IAA* genes was therefore investigated by qRT–PCR in seedling tissues. All of the *Aux/IAAs* tested, except two (*Sl-IAA8* and *Sl-IAA27*), displayed positive regulation of their transcript accumulation by auxin (**Fig. 6A**), with some genes being slightly up-regulated (*Sl-IAA9* and *Sl-IAA26*) and others strongly induced (*Sl-IAA2*, *Sl-IAA17* and *Sl-IAA19*). The analysis of ethylene regulation of tomato *Sl-IAA* genes in etiolated seedlings indicated that some genes were up-regulated while others were clearly down-regulated by ethylene (**Fig. 6B**). The data indicated that *Sl-IAA29* was strongly up-regulated, *Sl-IAA3* and *Sl-IAA36* were slightly up-regulated, and transcript accumulation of *Sl-IAA2*, *Sl-IAA11*, *Sl-IAA17* and *Sl-IAA19* genes was dramatically reduced upon ethylene treatment. These data suggest that in addition of being major molecular players in the auxin responses, some Aux/IAAs may also be potential components of the ethylene response.

Discussion

The comprehensive identification and subsequent characterization of the tomato *Aux/IAA* gene family members described here provide new insight regarding the potential role of some *Aux/IAA* genes in mediating plant responses to both auxin and ethylene. Moreover, by assessing the transcriptional repression

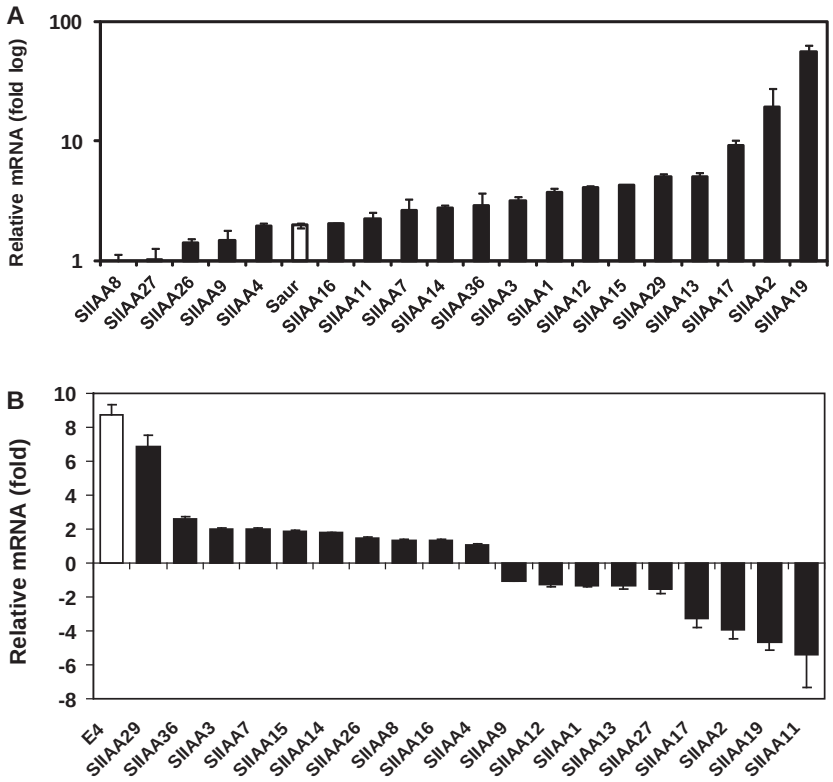

Fig. 6 Auxin and ethylene regulation of *Aux/IAA* genes in tomato. (A) Relative auxin induction of *Sl-IAA* genes in light-grown seedlings. Quantitative RT–PCR was used to assess *Sl-IAA* transcript accumulation in RNA samples extracted from 12-day-old tomato seedlings soaked in liquid MS medium with 10 μM IAA for 2 h. ΔΔCT refers to the fold difference in *IAA* expression compared with the untreated seedlings. The *SAUR* gene was used as a control to validate the auxin treatment. The vertical axis is displayed on a logarithmic scale to obtain a better comparison of transcript levels. (B) Ethylene regulation of *Sl-IAA* genes on dark-grown seedlings. Quantitative RT–PCR of *Sl-IAA* transcripts in RNA samples extracted from 5 d dark-grown tomato seedlings treated for 5 h with ethylene (50 μl l^{-1}). ΔΔCT refers to fold differences in *IAA* expression relative to untreated seedlings. The *E4* gene was used as control for efficient ethylene treatment.

capacity, the spatio-temporal expression patterns and the subcellular localization at the protein level, this study provides new leads towards addressing the putative function and mode of action of tomato *Aux/IAA* genes. The tomato *Aux/IAA* family is slightly contracted, with 25 members compared with Arabidopsis (29 genes) (Liscum and Reed 2002). However, while overall the tomato *Aux/IAA* gene family comprises a lower number of genes than in Arabidopsis, two clades are substantially expanded. Clades A and J contain seven and three genes in tomato, respectively, but only four and one in Arabidopsis. As an illustration of the wide diversification of Aux/IAA proteins in higher plants, the two clades are also expanded in *Populus trichocarpa*, with six members in clade A and three members in clade J (Kalluri et al. 2007). This diversification is also reflected by important structural variations found within Aux/IAA proteins. The accepted model for Aux/IAA function builds on auxin-mediated degradation of these short-lived proteins that typically have four conserved domains defining the gene family members. Notably, clade H comprising three non-canonical members (AtIAA20, AtIAA30 and AtIAA31) in Arabidopsis that lack the conserved domain II essential for protein degradation is not represented in tomato. In line with the absence or the alteration of domain II, AtIAA20 and AtIAA31 have been shown to be long-lived proteins compared with the canonical AtIAA17 (Dreher et al. 2006). The mechanism by which these non-canonical proteins impact auxin signaling remains unclear, even though the overexpression of *AtIAA20*, *AtIAA30* or *AtIAA31* results in aberrant auxin-related phenotypes in Arabidopsis (Sato and Yamamoto 2008). The tomato genome contains two non-canonical *Aux/IAA* genes (*Sl-IAA32* and *Sl-IAA33*), whereas up to six are found in Arabidopsis. Sl-IAA32 protein lacks domain II, whereas both domain I and domain II are missing in Sl-IAA33. The present study shows that Sl-IAA32 is a functional repressor of auxin signaling and its expression is limited to the breaker stage of fruit development (data not shown). A search in the SGN database identified an EST sequence from a cell culture suspension corresponding to *Sl-IAA33*, suggesting that the expression of this gene is highly constrained. Attempts to detect *Sl-IAA33* mRNA in the present study were unsuccessful in all tissues tested, further supporting the low level of expression of non-canonical *Aux/IAA* genes reported so far in Arabidopsis (Dreher et al. 2006). Considering their expression pattern apparently restricted to narrow developmental stages and their atypical long-lived feature due to the absence of domain II, the tomato non-canonical Aux/IAA proteins may have a specific function in mediating auxin responses during well-defined plant developmental events.

The expression patterns of *Sl-IAA* genes in various tissues and organs suggest that the encoded proteins may perform both specific and redundant functions. Nevertheless, no link was found between the clustering based on the expression pattern and the clustering obtained by phylogenetic analysis, with genes from the same clade, such as clade A, displaying either a high (*Sl-IAA3* and *Sl-IAA4*) or a low (*Sl-IAA2* and *Sl-IAA22*) level of expression. For the two remaining members of clade A (*Sl-IAA21* and *Sl-IAA23*) no corresponding EST was found in the databases, and attempts to detect the corresponding mRNAs failed in all tissues tested. The six members of the analog clade in *P. trichocarpa* (*PtIAA3* subgroup) are differentially transcribed (Kalluri et al. 2007) and, likewise, in Arabidopsis, gene expression patterns of Aux/IAA sister pairs are significantly different (Paponov et al. 2009). These data support the idea that the diversification of Aux/IAA family members in flowering plants has also been sustained by changes in their expression patterns. The majority of *Sl-IAA* genes identified are transcriptionally active as assessed by the isolation of the full-length open reading frame corresponding to 22 genes out of the 25 members present in the tomato genome. For most *Aux/IAA* genes, the highest expression level was found in young leaves and seedlings, two tissues known to accumulate a high amount of auxin. The transcript levels of 17 out of 19 *Sl-IAA* genes were up-regulated by auxin treatment in seedlings, though to varying degrees. Consistent with this high degree of regulation by auxin, promoter analysis revealed the presence of well-conserved AuxREs in the promoter region of the majority of *Sl-IAA* genes. Members of the Arabidopsis *Aux/IAA* gene family have also been shown to respond to exogenous IAA in a highly differential fashion with respect to dosage and time (Abel et al. 1994, Abel et al. 1995). A variety of factors may explain the differences observed in the response kinetics between individual *Aux/IAA* genes such as tissue-specific auxin perception, cell type dependence and differential regulation of free auxin concentrations, or different modes of auxin-dependent transcriptional and post-transcriptional regulation. It has been reported previously that down-regulation of *Sl-IAA3* results in auxin- and ethylene-related developmental defects including reduced apical dominance, reduced auxin response and an exaggerated apical hook in etiolated seedlings (Chaabouni et al. 2009a), supporting the hypothesis that *Sl-IAA3* represents a molecular link between ethylene and auxin signaling in tomato (Chaabouni et al. 2009b). Ethylene responsiveness of *Aux/IAA* genes was first described in late immature green tomato fruit (Jones et al. 2002). The present study provides a more comprehensive analysis of the ethylene regulation of *Sl-IAA* genes, revealing that the expression of some genes is clearly and rapidly induced by ethylene in etiolated seedlings, with *Sl-IAA29* transcript accumulation being the most strongly up-regulated. In contrast, ethylene treatment dramatically reduced transcript accumulation of *Sl-IAA2*, *Sl-IAA11*, *Sl-IAA17* and *Sl-IAA19* genes. Strikingly, none of these ethylene-regulated tomato *Aux/IAA* genes contains the conserved GCC-box motif, a *cis*-acting element present in the promoter regions of ethylene-responsive genes (Ohme-Takagi and Shinshi 1995). Notably, five out of seven ethylene-regulated *Sl-IAA* genes contain another ethylene-response motif, the so-called ERELEE4 motif (AWTTCAAA), found in the promoter of the tomato *E4* gene, a well-described ripening- and ethylene-regulated gene (Montgomery et al. 1993). The potential role of the ethylene-regulated *Aux/IAA* genes in mediating

the cross-talk between auxin and ethylene remains to be further investigated, in particular during developmental events such as apical hook formation or the transition from green to ripe fruit where ethylene is known to be a key player.

The nuclear targeting of tomato Aux/IAA proteins is consistent with a transcriptional regulatory function. Typical Aux/IAA proteins harbor two NLSs, one bipartite and one resembling an SV40-type NLS. In tomato, all the Sl-IAAs tested so far localize in the nuclear compartment (Wang et al. 2005, Chaabouni et al 2009a, Deng et al., 2012). While the present study confirms the nuclear targeting of some other members of the AUX/IAA proteins (Sl-IAA4, Sl-IAA22 and Sl-IAA29), it also reveals the presence of Sl-IAA32 protein, which lacks the bipartite NLS, in both the nucleus and the cytoplasm. The lack of a bipartite NLS in the native Sl-IAA32 protein is likely to be responsible for the targeting of this protein to the extranuclear compartment. These data suggest that some Aux/IAA proteins may have an extranuclear function that still remains to be elucidated. It is important to mention that, in addition to its cytoplasmic localization, Sl-IAA32 also lacks the conserved domain II required for the degradation of the protein mediated by the auxin–TIR1 complex, thus raising the hypothesis that this Aux/IAA may be involved in a mechanism independent from the conventional auxin signaling pathway.

In agreement with previous reports, all tomato Aux/IAAs displayed a repression activity of auxin-dependent transcription (Ulmasov et al. 1997, Tiwari et al. 2001, Bargmann and Birnbaum 2009). However, the repression levels vary widely (23–87%) among tomato Aux/IAA proteins when tested with the synthetic DR5 promoter. It has been previously described in Arabidopsis that domain I of Aux/IAA proteins is an active, portable repression domain containing the LxLxL motif (Tiwari et al. 2004) that interacts with the TOPLESS (TPL) co-repressor (Szemenyei et al. 2008). All the tomato Aux/IAAs tested in this study bear a conserved domain I, but no correlation was found between the level of repression and the amino acid environment surrounding the LxLxL repressor motif. Interestingly, Sl-IAA26, showing the strongest repression activity, contains, in addition to the LxLxL motif, a second LxLxPP motif, found in *Physcomitrella patens* and other flowering plants, that has been proposed to function as a putative repression domain (kk**Le**L**r**L**g****PP**) (Paponov et al. 2009). Sl-IAA26 belongs to clade F with three other Arabidopsis Aux/IAAs (AtIAA18, AtIAA26 and AtIAA28) also containing this overlapping LxLxLxPP motif. Yet, the potential of the LxLxPP motif to potentiate the repressor activity of Aux/IAA proteins is not supported by any direct experimental evidence. Recently, it has been reported that mutations in domain I of various Aux/IAA proteins can have profound, but different, consequences in terms of auxin responses in Arabidopsis plants, suggesting that some Aux/IAA proteins may have stronger or more complex repression domains than others (Li et al. 2011). However, in tomato, neither the length of the repression domains (e.g. an LxLxL vs. an LxLxLxLxL motif) nor the presence of two LxLxL motifs in the same Aux/IAA protein seems to correlate with the level of transcriptional repression of the synthetic DR5 or the native *Sl-IAA3* promoter. Dedicated tomato mutant resources are now needed to better understand the intrinsic differences in the repression domains of Sl-IAA proteins and to better clarify the functional significance of the diversification of Aux/IAA members between tomato and Arabidopsis. Moreover, to understand the functional differentiation among the Aux/IAA family in tomato will also require the determination of qualitative and quantitative interactions between Aux/IAAs and their ARF partners. It should also taken be into consideration in future studies that several lines of evidence in the literature support a model for EAR motif-mediated repression acting via epigenetic mechanisms resulting from chromatin modifications (Kagale and Rozwadowski 2011).

Materials and Methods

Plant material and growth conditions

Tomato seeds (*S. lycopersicum* cv. MicroTom or Ailsa Craig) were sterilized, rinsed in sterile water and sown in recipient Magenta vessels containing 50 ml of 50% Murashige and Skoog (MS) culture medium with added R3 vitamin (0.5 mg l^{-1} thiamine, 0.25 mg l^{-1} nicotinic acid and 0.5 mg l^{-1} pyridoxine), 1.5% (w/v) sucrose and 0.8% (w/v) agar, pH 5.9. Plants were grown under standard greenhouse conditions. The culture chamber rooms were set as follows: 14 h day/10 h night cycle, 25/20°C day/night temperature, 80% relative humidity and 250 µmol m^{-2} s^{-1} intense luminosity.

Transient expression using a single cell system

Protoplasts for transfection were obtained from suspension-cultured tobacco (*Nicotiana tabacum*) BY-2 cells according to the method described previously (Leclercq et al. 2005). Protoplasts were transfected by a modified polyethylene glycol method as described by Abel and Theologis (1994). For nuclear localization of the selected Aux/IAA fusion proteins, the coding sequences of genes were cloned as a C-terminal fusion in-frame with GFP or as an N-terminal fusion with YFP under the control of the 35S CaMV promoter. Transfected protoplasts were incubated for 16 h at 25°C and analyzed for GFP/YFP fluorescence by confocal microscopy. Confocal imaging was conducted on a Leica TCS SP2 confocal laser scanning microscope. Images were obtained with a ×40 1.25 numerical aperture water-immersion objective. GFP and YFP were excited at 488 nm, and the emitted light was captured at 505–535 nm and 530–570 nm, respectively. For co-transfection assays, aliquots of protoplasts (0.5×10^6) were transformed either with 10 µg of the reporter vector alone containing the promoter fused to the GFP reporter gene or in combination with 10 µg of Aux/IAA construct as the effector plasmid. Transformation assays were performed in three independent replicates. After 16 h, GFP expression was analyzed and quantified by flow cytometry (FACS Calibur II instrument, BD Biosciences) on a flow cytometry platform

(IRF31). Data were analyzed using Cell Quest software. For each sample, 100–1,000 protoplasts were gated on forward light scatter and the GFP fluorescence per population of cells corresponds to the average fluorescence intensity of the cell population after subtraction of autofluorescence determined with non-transformed BY-2 protoplasts. The data were normalized using an experiment, in the presence of 50 μM 2,4-D, with protoplasts transformed with the reporter vector in combination with the vector used as the effector plasmid but lacking the *Sl-IAA* coding region.

RNA isolation and qRT–PCR

Total RNA was extracted from fruit according to Hamilton et al. (1990). Total RNA from leaves and seedlings was extracted using a Plant RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Total RNA was treated by DNase I to remove any genomic DNA contamination. First-strand cDNA was reverse transcribed from 2 μg of total RNA using an Omniscript kit (Qiagen) according to the manufacturer's instructions. qRT–PCR analyses were performed as previously described (Pirrello et al. 2006). The primer sequences are listed in **Supplementary Table S4**. Relative fold differences were calculated based on the comparative Ct method using Sl-Actin-51 as an internal standard. To determine relative fold differences for each sample in each experiment, the Ct value of genes was normalized to the Ct value for Sl-Actin-51 (accession No. Q96483) and was calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$. At least two to three independent RNA isolations were used for cDNA synthesis and each cDNA sample was subjected to real-time PCR analysis in triplicate. Heat map representation was performed using centring and the normalized ΔCt value, with Cluster 3.0 software and JavaTreeview to visualize the dendogram.

Hormone treatment

For auxin treatment on light-grown seedlings, 12-day-old Ailsa Craig seedlings (30 seedlings) were soaked in liquid MS medium with or without (mock treatment) 10 μM IAA for 2 h. The efficiency of the treatment was checked by measuring the induction of the tomato early auxin-responsive *SAUR* gene. For ethylene treatment on dark-grown seedlings, 5-day-old MicroTom seedlings (100 seedlings) were treated with air or ethylene gas (50 μl l^{-1}) for 5 h. The efficiency of the treatment was checked by measuring the induction of the tomato ethylene-responsive *E4* gene. The experiment was repeated with three biological replicates.

Sequence data for the Arabidopsis genes used in this article can be found in the Arabidopsis Genome Initiative data library under the following accession numbers: *AtIAA1* (AT4G14560), *AtIAA2* (AT3G23030), *AtIAA3* (AT1G04240), *AtIAA4* (AT5G43700), *AtIAA5* (AT1G15580), *AtIAA6* (AT1G52830), *AtIAA7* (AT3G23050), *AtIAA8* (AT2G22670), *AtIAA9* (AT5G65670), *AtIAA10* (AT1G04100), *AtIAA11* (AT4G28640), *AtIAA12* (AT1G04550), *AtIAA13* (AT2G33310), *AtIAA14* (AT4G14550), *AtIAA15* (AT1G80390), *AtIAA16* (AT3G04730), *AtIAA17* (AT1G04250), *AtIAA18* (AT1G51950), *AtIAA19* (AT3G15540), *AtIAA20* (AT2G46990), *AtIAA26* (AT3G16500), *AtIAA27* (AT4G29080), *AtIAA28* (AT5G25890), *AtIAA29* (AT4G32280), *AtIAA30* (AT3G62100), *AtIAA31* (AT3G17600), *AtIAA32* (AT2G01200), *AtIAA33* (AT5G57420), *AtIAA34* (AT1G15050).

Supplementary data

Supplementary data are available at PCP online

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Supplementary Tables

Table S1. Gene name, SGN number, EST number, ITAG release 2.3 number, chromosome localisation (K), and *cis*-elements in promoter (ARFAT, ARFATdg, ERELEE4) of the tomato *Aux/IAA* gene family members.

Gene name	SGN Unigene	EST number in Unigene	ITAG Release 2.3	K	ARFAT	ARFATdg	ERELEE4
Sl-IAA1	SGN-U579410	10	Solyc09g083280.2.1	chr09	0	3	1
Sl-IAA2	SGN-U599474	1	Solyc06g084070.2.1	chr06	0	1	0
Sl-IAA3	SGN-U577993	17	Solyc09g065850.2.1	chr09	0	2	5
Sl-IAA4	SGN-U579749	36	Solyc06g053840.2.1	chr06	0	2	2
Sl-IAA7	SGN-U579168	15	Solyc06g053830.2.1	chr06	2	0	1
Sl-IAA8	SGN-U581702	11	Solyc12g007230.1.1	chr12	1	2	0
Sl-IAA9	SGN-U568849	104	Solyc04g076850.2.1	chr04	1	0	4
Sl-IAA11	SGN-U577813	5	Solyc12g096980.1.1	chr12	3	0	1
Sl-IAA12	SGN-U579795	8	Solyc09g064530.2.1	chr09	1	1	0
Sl-IAA13	SGN-U579354	9	Solyc09g090910.1.1	chr09	0	2	3
Sl-IAA14	SGN-U579618	20	Solyc09g083290.2.1	chr09	0	0	0
Sl-IAA15	SGN-U579568	28	Solyc03g120390.2.1	chr03	1	2	4
Sl-IAA16	SGN-U580151	6	Solyc01g097290.2.1	chr01	2	0	0
Sl-IAA17	SGN-U593495, SGN-U581524	1, 5	Solyc06g008590.2.1	chr06	2	0	0
Sl-IAA19	SGN-U579607	2	Solyc03g120380.2.1	chr03	1	1	2
Sl-IAA21		0		chr03	2	0	1
Sl-IAA22		0	Solyc06g008580.2.1	chr06	1	3	0
Sl-IAA23		0	Solyc04g054280.1.1	chr04	1	2	0
Sl-IAA26	SGN-U573372	13	Solyc03g121060.2.1	chr03	5	0	1
Sl-IAA27	SGN-U577682, SGN-U580267	7, 7	Solyc03g120500.2.1	chr03	0	0	0

Sl-IAA29	SGN-U568970	13	Solyc08g021820.2.1	chr08	0	0	3
Sl-IAA32		0	Solyc05g008850.2.1	chr05	2	0	2
Sl-IAA33	SGN-U603679	1	Solyc07g019450.2.1	chr07	1	0	1
Sl-IAA35	SGN-U563561	5	Solyc07g008020.2.1	chr07	1	0	5
Sl-IAA36	SGN-U586760	1	Solyc06g066020.2.1	chr06	0	0	1

The numbers of *cis*-element in each 2kb promoter are given in the table (ARFAT corresponding to canonical auxin response element TGTCTC, ARFATdg corresponding to degenerated AuxRE TGTCCC, ERELEE4 corresponding to ethylene responsive element of tomato E4 gene AWTTCAAA).

Table S2. Predicted molecular mass, number of amino acids and isoelectric points of the tomato Aux/IAA protein family members.

Protein	No. of amino acids	Isoelectric pt	Predicted Mol. Mass (Da)
Sl-IAA1	196	5.86	22019
Sl-IAA2	156	8.62	17541
Sl-IAA3	185	6.81	20763
Sl-IAA4	190	6.26	21344
Sl-IAA7	218	9.01	24672
Sl-IAA8	295	8.50	32039
Sl-IAA9	349	6.99	37385
Sl-IAA11	233	6.99	26155
Sl-IAA12	313	9.01	33270
Sl-IAA13	283	8.63	30635
Sl-IAA14	236	7.69	26240
Sl-IAA15	252	9.08	28034
Sl-IAA16	251	8.26	27591
Sl-IAA17	208	8.86	23540
Sl-IAA19	192	6.04	21842
Sl-IAA21	213	6.28	24384
Sl-IAA22	190	6.08	21289
Sl-IAA23	166	8.84	19292
Sl-IAA26	287	9.05	31923
Sl-IAA27	278	7.06	30544
Sl-IAA29	227	8.67	26719
Sl-IAA32	188	5.02	22038
Sl-IAA33	147	6.91	16404
Sl-IAA35	196	7.96	22313
Sl-IAA36	242	5.22	28210

Table S3. Amino acid sequence comparison of the tomato Aux/IAA proteins

Numbers denote the percentage of identity between predicted proteins. Values that showed high identity are shaded

	IAA4	IAA21	IAA23	IAA2	IAA22	IAA3	IAA11	IAA13	IAA12	IAA26	IAA29	IAA36	IAA33	IAA32	IAA35	IAA14	IAA7	IAA16	IAA17	IAA15	IAA27	IAA9	IAA8	IAA19
IAA1	78	72	67	63	58	58	37	35	35	34	24	28	23	24	28	50	49	48	53	49	48	47	49	47
IAA4		70	65	61	59	58	40	40	36	33	25	27	22	24	29	51	48	48	51	44	48	46	47	49
IAA21			79	60	54	57	36	34	32	34	23	26	22	24	28	49	46	45	51	42	44	43	44	46
IAA23				60	56	54	41	39	40	36	26	28	24	26	30	46	48	49	51	46	48	45	46	49
IAA2					64	67	43	42	44	38	28	33	25	26	38	56	58	58	59	56	57	54	54	55
IAA22						64	40	36	38	32	27	30	21	23	33	48	50	49	50	47	50	48	45	44
IAA3							38	35	36	35	26	29	24	23	33	52	53	50	55	49	50	50	50	49
IAA11								61	46	32	24	25	24	31	30	38	37	36	46	41	42	39	38	39
IAA13									38	30	21	23	22	29	27	39	38	33	39	33	36	33	35	33
IAA12										29	23	24	20	26	30	36	36	33	40	30	37	32	35	37
IAA26											24	23	22	22	26	36	36	33	36	36	32	32	32	32
IAA29												52	26	24	33	28	31	24	29	28	24	24	24	27
IAA36													27	24	36	26	28	23	26	26	24	24	25	25
IAA33														21	23	22	23	21	23	24	19	20	19	22
IAA32															24	26	26	24	27	27	25	24	22	25
IAA35																32	33	33	34	36	33	30	32	30
IAA14																	75	71	70	58	54	54	51	46
IAA7																		68	72	57	53	56	52	47
IAA16																			69	55	51	51	49	49
IAA17																				62	56	56	54	48
IAA15																					50	46	50	44
IAA27																						56	56	48
IAA9																							53	42
IAA8																								44

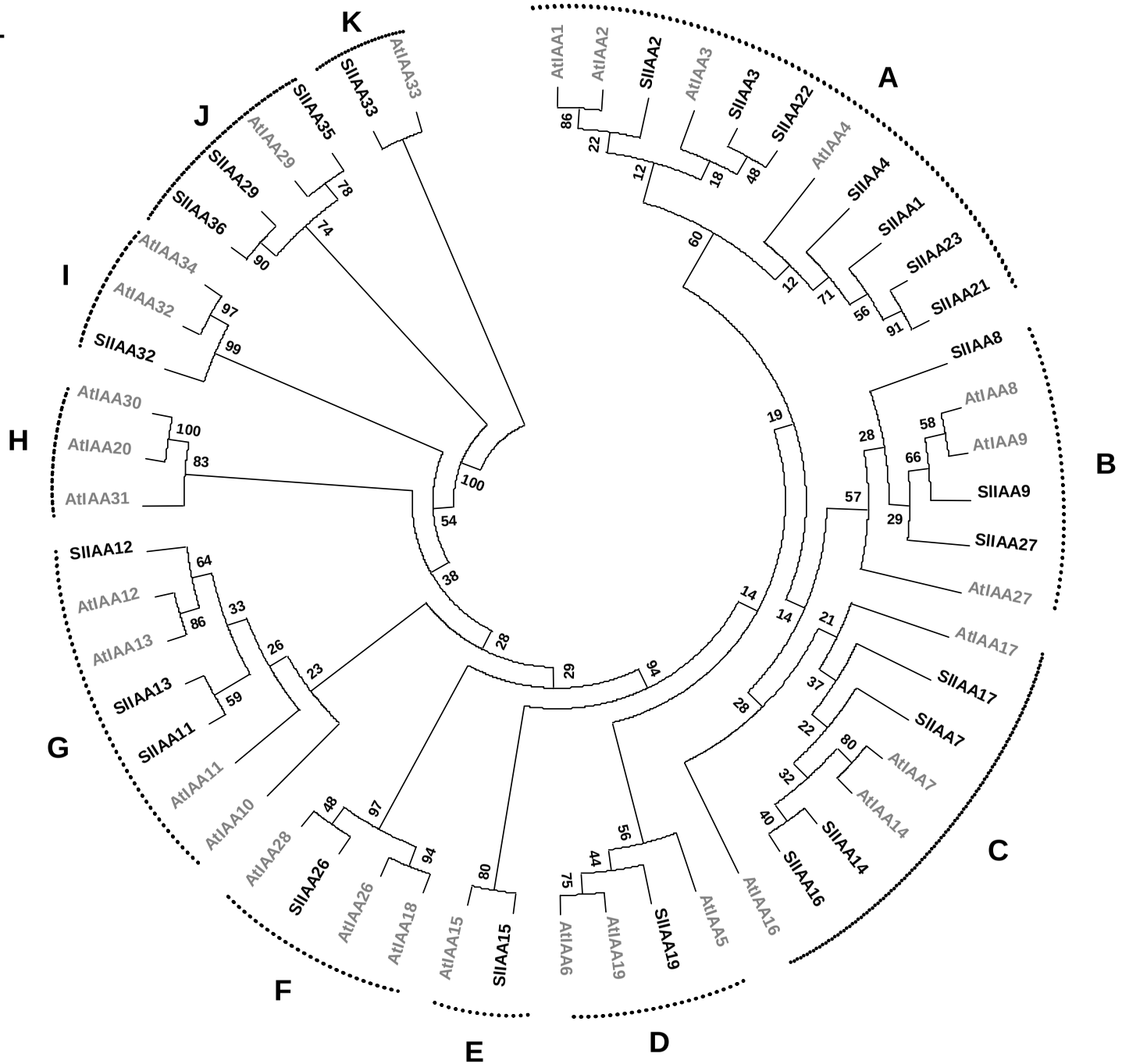
Table S4. Aux/IAA quantitative PCR primers

The accession numbers and primer-sequences of the genes described in this article are listed below.

Gene name	Primer Sequence	SGN number
Actin	F 5'-TGTCCCTATCTACGAGGGTTATGC-3'	SGN-U580609
	R 5'-AGTTAAATCACGACCAGCAAGAT-3'	
Sl-IAA1	F 5'-TGAATCTAAGTCAAGTTCTGATCATGTC-3'	SGN-U579410
	R 5'-ATGATGTTTTTCCTGTTAGATCTCACTG-3'	
Sl-IAA2	F 5'-TAACAATGATGAACCACCAC-3'	SGN-U599474
	R 5'-TTTCCTTAAATAAGCCGCAC-3'	
Sl-IAA3	F 5'-ATATAATGGATCTGATTATGCACCAACA-3'	SGN-U577993
	R 5'-TTATAAACATCTCCCATGGTACATCAC-3'	
Sl-IAA4	F 5'-AACAAGAGGGCTTTGCCTGAG-3'	SGN-U579749
	R 5'-GTGTCTTGGCAACAGGTGGA-3'	
Sl-IAA7	F 5'-ACTCAACCTCCATCATAATGATAATATTCC-3'	SGN-U579168
	R 5'-ACCCCACCACTTGAGCCTTA-3'	
Sl-IAA8	F 5'-ATTCTGCTACTTTGATAATCTTGCACA-3'	SGN-U581702
	R 5'-TGTCCATTGATGAAACACAGCTCT-3'	
Sl-IAA9	F 5'-CCCCTTGCACCCTTCCA-3'	SGN-U568849
	R 5'-AGCGTCTGAAAATCCTCGTTTG-3'	
Sl-IAA11	F 5'-GGAGATGTTCTTCAAATCAACC-3'	SGN-U577813
	R 5'-TCTGATGATCCATCCAAGAG-3'	
Sl-IAA12	F 5'-CCACGCGATCTTCAGCATAA-3'	SGN-U579795
	R 5'-TCTGTTTCAGGGAGCGGC-3'	
Sl-IAA13	F 5'-AGTCTTTTAAGCTCTTGGATGGATCA-3'	SGN-U579354
	R 5'-AAACATCCCGAATGGAACATCT-3'	
Sl-IAA14	F 5'-GTTTACGCATAATGAAAGGATCAGAAG-3'	SGN-U579618
	R 5'-TTATCTATGGAGCTTGCACACCA-3'	
Sl-IAA15	F 5'-CCTAACAATCTGTAATTCTCAAAGTGAAA-3'	SGN-U579568
	R 5'-GCATCCAGTCTCCATCTTTATCTTC-3'	
Sl-IAA16	F 5'-GCGTGTTGGGTGCGGA-3'	SGN-U580151
	R 5'-CGATTCCAGTTCATTCCCATTAG-3'	
Sl-IAA17	F 5'-CAAGAATTATTTGATGCCTTAACCAA-3'	SGN-U593495 SGN-U581524

	R 5'-ACTATTCAAAAGGTCCATCAGTTTCC-3'	
Sl-IAA19	F 5'-TGTCGGCGATGTTCCATG-3'	SGN-U579607
	R 5'-AAGTCTCTTGCTCCAAGCCCTAT-3'	
Sl-IAA26	F 5'-AAAGGCTGCGTGTGTTGAAA-3'	SGN-U573372
	R 5'-CAAGATCTGTTGGCTCTACATCTTGT-3'	
Sl-IAA27	F 5'-CCAAAAAGAGGGAATGGAGGTT-3'	SGN-U577682 SGN-U580267
	R 5'-TGTTCTCCCTTCATCATCATTTTTC-3'	
Sl-IAA29	F 5'-GGTTTTGATGATAGCTTCTCCGATA-3'	SGN-U568970
	R 5'-ACGTCTTACGTTCAACTACTCCTTCA-3'	
Sl-IAA36	F 5'-AGAAATTCCATGTATGTGAAGGTTAAAA-3'	SGN-U586760
	R 5'-CATTTGGAGCAAAGTGTTAGTAAGGA-3'	
Saur	F 5'-GCCTCAGATCCTATTCAAGCGA-3'	SGN-U563523
	R 5'-TCCAATGGGCGGTGTCA-3'	
E4	F 5'-CTACTACCCACCATGTCCTCAGC-3'	SGN-U578448
	R 5'-AAGGATCGTTACAAAACCAATATCG-3'	

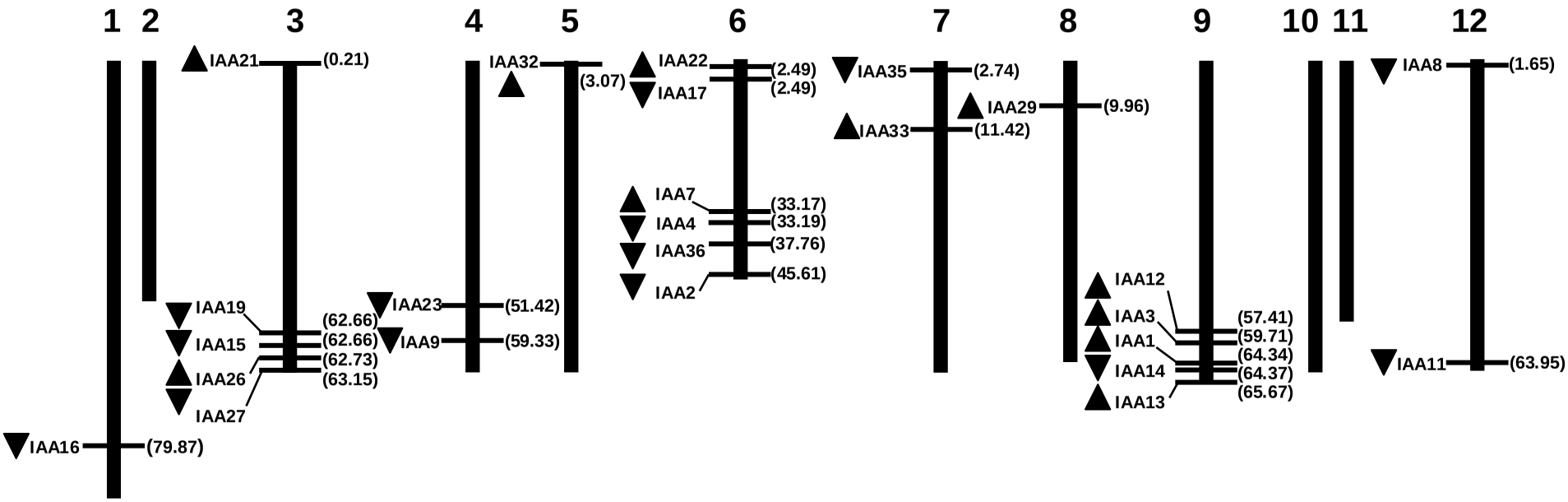
FigS1


A
B
C
D
E
F
G
H
I
J
K
AtIAA1
AtIAA2
SlIAA2
AtIAA3
SlIAA3
SlIAA22
AtIAA4
SlIAA4
SlIAA1
SlIAA23
SlIAA21
SlIAA8
AtIAA8
AtIAA9
SlIAA9
SlIAA27
AtIAA27
AtIAA17
SlIAA17
SlIAA7
AtIAA7
AtIAA14
SlIAA14
SlIAA16
AtIAA16
AtIAA5
SlIAA19
AtIAA19
AtIAA6
AtIAA15
SlIAA15
AtIAA18
AtIAA26
SlIAA26
AtIAA28
AtIAA10
AtIAA11
SlIAA11
SlIAA13
AtIAA13
AtIAA12
SlIAA12
AtIAA31
AtIAA20
AtIAA30
SlIAA32
AtIAA32
AtIAA34
SlIAA36
SlIAA29
AtIAA29
SlIAA35
SlIAA33
AtIAA33

Supplemental Figure S1. Evolutionary relationships of Arabidopsis and tomato Aux/IAA proteins.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 7.80811039 is shown. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved 54 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 68 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

FigS2

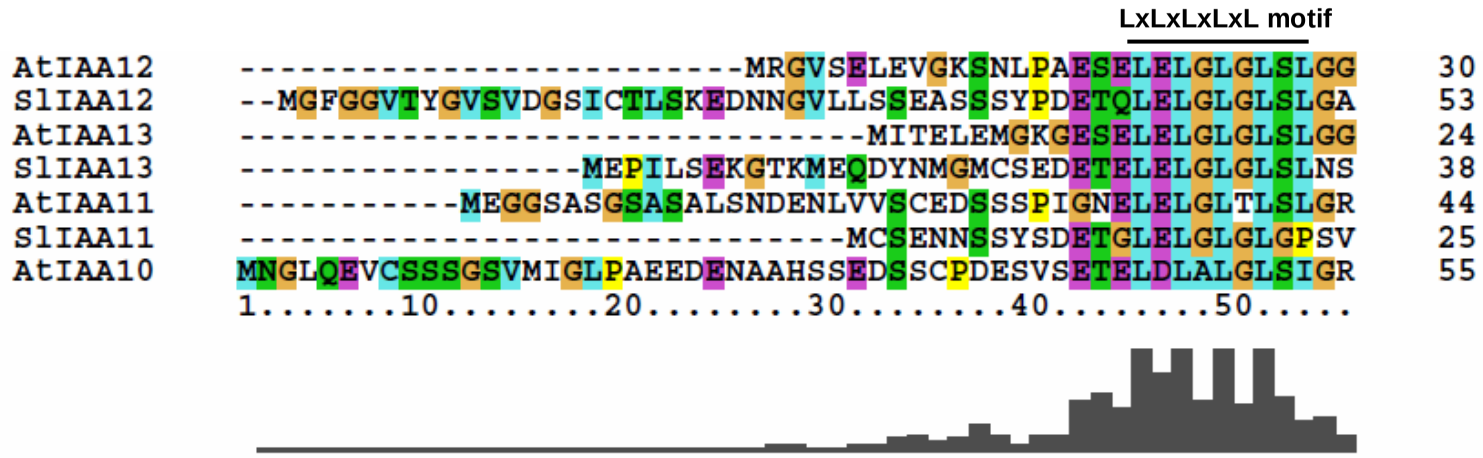


Supplemental Figure S2. Genomic distribution of *Aux/IAA* genes on tomato chromosomes.

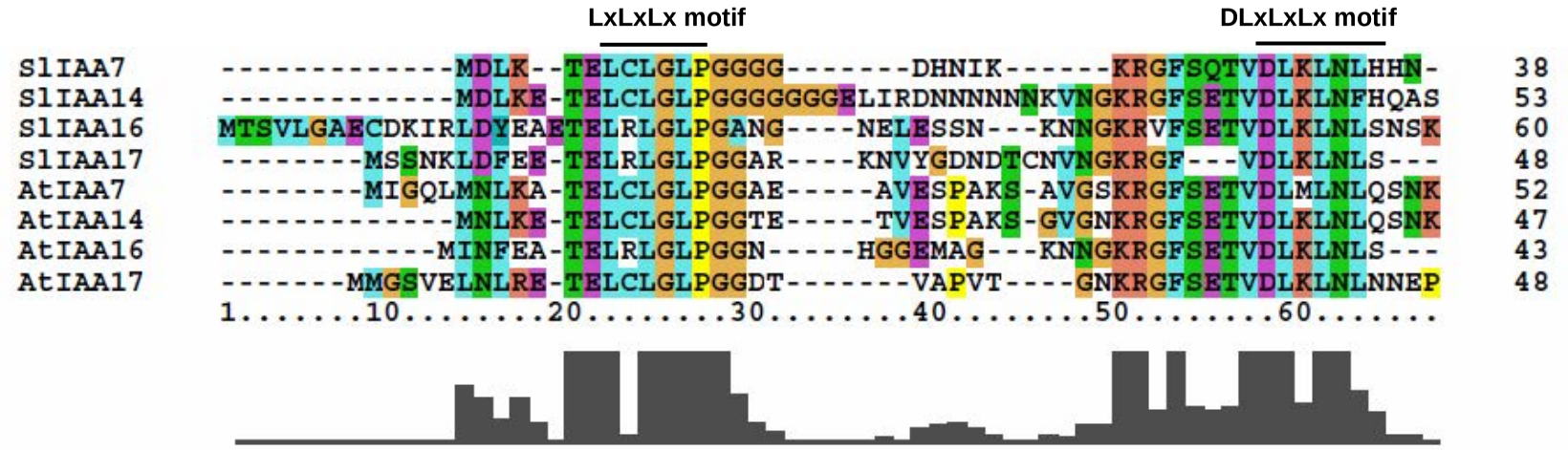
The arrows next to gene names show the direction of transcription. The number in parentheses designates the position of the ATG of each *Aux/IAA* gene in megabases (Mb) on tomato chromosome pseudomolecules (Tomato Whole Genome Scaffolds data V2.4). The chromosome numbers are indicated at the top of each bar.

FigS3

A


LxLxLxLxL motif
AtIAA12 ----------MRGVSELEVGKSNLPAESELELGLGLSLGG 30
SlIAA12 --MGFGGVTYGVSVDGSICTLSKEDNNGVLLSSEASSSYPDETQLELGLGLSLGA 53
AtIAA13 ----------MITELEMGKGESELELGLGLSLGG 24
SlIAA13 ----------MEPILSEKGTKMEQDYNMGMCSEDETELELGLGLSLNS 38
AtIAA11 ----------MEGGSASGSASALSNDENLVVSCEDSSSPIGNELELGLTLSLGR 44
SlIAA11 ----------MCSENNSSYSDETGLELGLGLGPSV 25
AtIAA10 MNGLQEVCSSSGSVMIGLPAEEDENAAHSSEDSSCPDESVSETELDLALGLSIGR 55
1.....10.....20.....30.....40.....50.....

B


LxLxLx motif
DLxLxLx motif
SlIAA7 ----------MDLK--TELCLGLPGGGG-------DHNIK------KRGFSQTVDLKLNLHHN- 38
SlIAA14 ----------MDLKE-TELCLGLPGGGGGGGELIRDNNNNNNKVNGKRGFSETVDLKLNFHQAS 53
SlIAA16 MTSVLGAECDKIRLDYEAETELRLGLPGANG----NELESSN---KNNGKRVFSETVDLKLNLSNSK 60
SlIAA17 --------MSSNKLDFEE-TELRLGLPGGAR----KNVYGDNDTCNVNGKRGF---VDLKLNLS--- 48
AtIAA7 --------MIGQLMNLKA-TELCLGLPGGAE-----AVESPAKS-AVGSKRGFSETVDLMLNLQSNK 52
AtIAA14 ----------MNLKE-TELCLGLPGGTE-----TVESPAKS-GVGNKRGFSETVDLKLNLQSNK 47
AtIAA16 ----------MINFEA-TELRLGLPGGN-----HGGEMAG---KNNGKRGFSETVDLKLNLS--- 43
AtIAA17 -------MMGSVELNLRE-TELCLGLPGGDT-------VAPVT----GNKRGFSETVDLKLNLNNEP 48
1.....10.....20.....30.....40.....50.....60.....

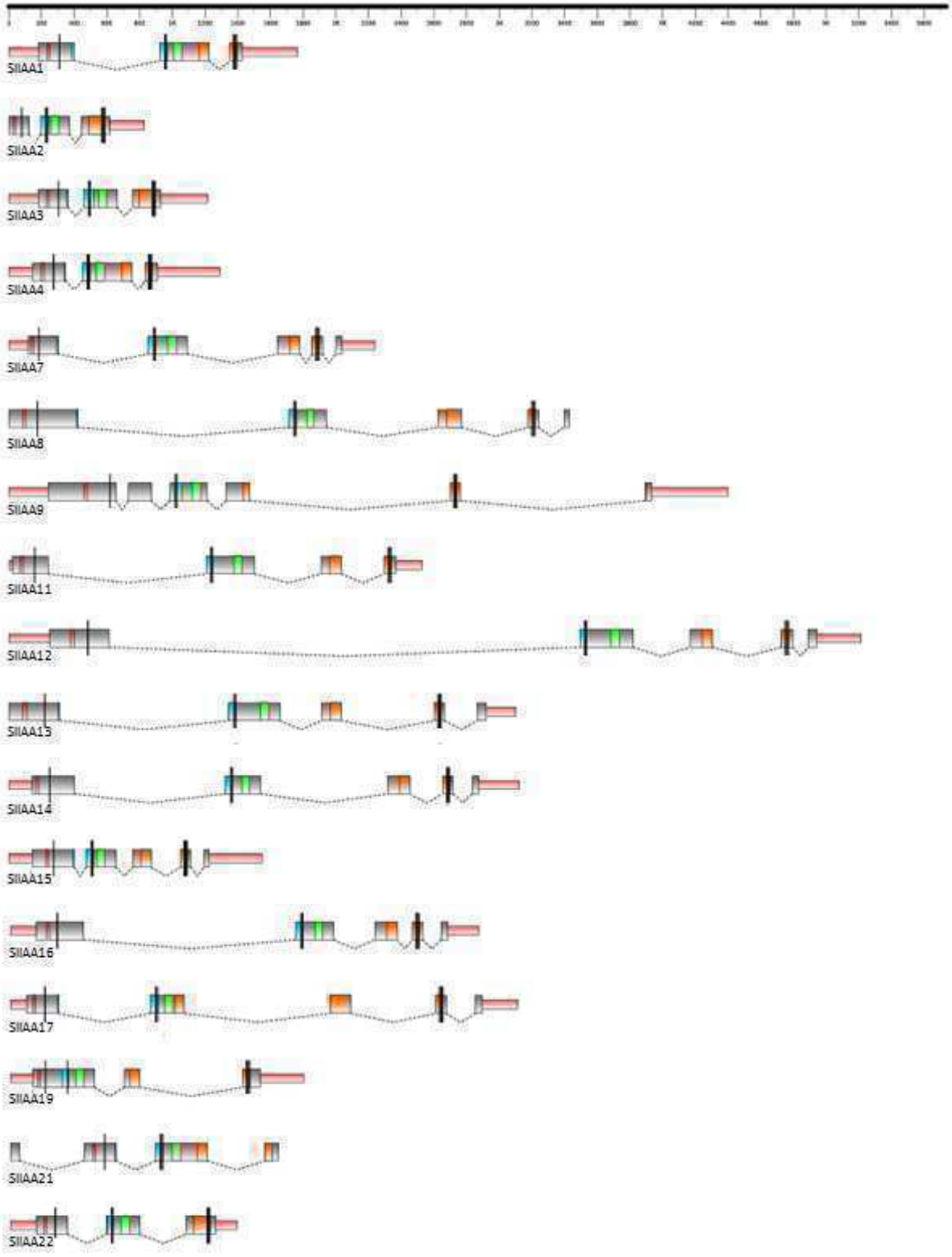
Supplemental Figure S3. Amino acid sequences for IAA proteins that contain an atypical domain I

(A) Amino acid sequences for IAA proteins that contain an expanded repression domain I. Amino acid sequence comparison of tomato Sl-IAA11, Sl-IAA12 and Sl-IAA13 with those from Arabidopsis AtIAA10 (AT1G04100), AtIAA11 (AT4G28640), AtIAA12 (AT1G04550) and AtIAA13 (AT1G04550). The conserved LxLxLxLxLx motif is underlined. The sequences were aligned with Clustal X method and adjusted manually.

(B) Amino acid sequences between conserved domains I and II for IAA proteins that contain a second LxLxL motif. Amino acid sequence comparison of tomato Sl-IAA7, Sl-IAA14, Sl-IAA16 and Sl-IAA17 with those from Arabidopsis AtIAA7 (AT3G23050), AtIAA14 (AT4G14550), AtIAA16 (AT3G04730) and AtIAA17 (AT1G04250). The conserved LxLxLx motif (Domain I) and the DLxLxL motif are underlined. The sequences were aligned with Clustal X method and adjusted manually.

III Conclusion

As described in the article, the *Aux/IAA* gene family is constituted by 25 members in the tomato. Additionnally, the structure (exons and introns numbers, unstranslated regions, conserved domains) of each *Sl-IAA* is presented in Figure 31. The genomic sequence of *Aux/IAA* is very short, less than 4kb, except for *Sl-IAA27* and *Sl-IAA9*. In addition *Aux/IAAs* display very few introns, between two and five according to gene (Figure 31). The data obtained regarding *Aux/IAAs* pattern of expression suggest a tissue-specific function for some of them. Indeed only eight *Aux/IAAs* seem well expressed in fruit tissue. In addition the expression of most of *Sl-IAA* can be regulated by both auxin and ethylene. This underlies the possibility that cross-talk between these two hormones is mediated through *Aux/IAA* regulation. It is of particular importance due to that ethylene is a key regulator of climacteric fruit ripening such as tomato. Therefore, understanding regulation and function of Sl-IAAs could improve knowledge about how auxin and ethylene both coordinate fruit development. To determine *Sl-IAA* function in tomato plant development either mutant analysis or study of transgenic generated plants can be used. Here, a strategy by reverse genetic approach has been chosen and generated plants available will be described in the next chapter.


SlIAA1
SlIAA2
SlIAA3
SlIAA4
SlIAA7
SlIAA8
SlIAA9
SlIAA11
SlIAA12
SlIAA13
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SlIAA15
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SlIAA19
SlIAA21
SlIAA22

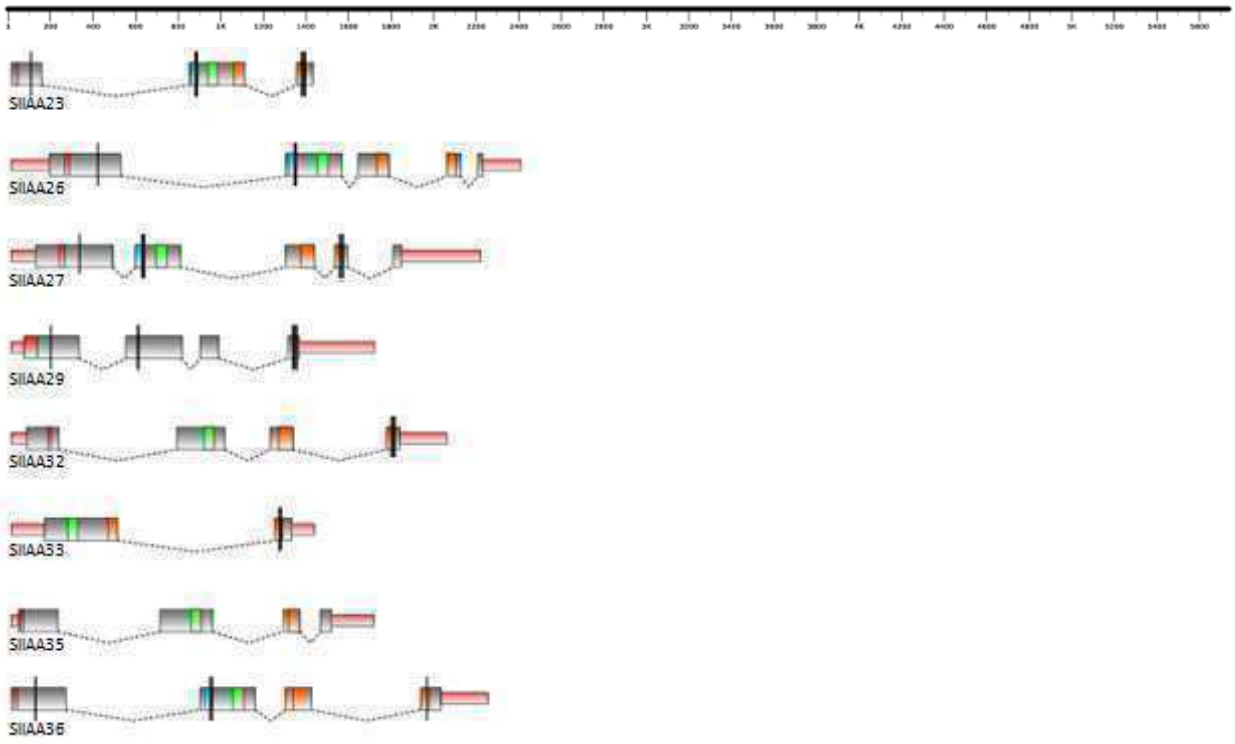

Figure 29: SlAux/IAAs structure designed with the fancyGENE software (http://host13.bioinfo3.ifom-ieo-campus.it/fancygene/). Exons are represented by grey boxes, introns by hats, untranslated regions by pink boxes, domain I by red box, domain II by blue box, domain III by green box and domain IV by amber box. Nuclear localization signals are represented by black vertical straights.

Chapter III: Analysis of Aux/IAA function in tomato, physiological significance of the *Sl-IAA27* gene

Résumé du chapitre III

Le chapitre III est consacré à l'étude fonctionnelle des *Aux/IAA*. Dans le cadre de ma thèse, je me suis focalisée sur la caractérisation phénotypique de 4 types de lignées transgéniques de type RNAi (*SlIAA16*, *SlIAA17*, *SlIAA27* et *SlIAA29)*. Ces analyses ont concernées en particulier la morphogénèse des fleurs, la mise à fruit et les caractéristiques et qualités du fruit (forme, couleur, texture, sucres,…). Le résultat le plus marquant a été obtenu dans le cas des lignées Sl-*IAA27* RNAi. En effet, ces lignées présentent une altération du développement des fruits. La majeure partie de ce chapitre est consacrée à la présentation des résultats obtenus quant à la caractérisation de ce gène principalement sous la forme d'une publication acceptée au journal Plant and Cell Physiology. Une analyse structurelle de *Sl-IAA27* a été réalisée montrant que la protéine encodée par ce gène possède les domaines conservés caractéristiques de la famille des *Aux/IAA*. Sl-IAA27 présente une localisation nucléaire et agit en tant que répresseur de l'activité transcriptionnelle dépendante de l'auxine. Les plantes sous-exprimant le gène *Sl-IAA27* ont montrées une diminution de la teneur en chlorophylle au niveau des feuilles, une modification de l'anatomie des fleurs et des fruits ainsi qu'une diminution du nombre de graines par fruit. Ces phénotypes ont mis en évidence la fonction de *Sl-IAA27* en tant que régulateur de la voie de biosynthèse des chlorophylles et du développement du fruit. Par ailleurs, la comparaison de la structure protéique de Sl-IAA27 avec celle de Sl-IAA9, son plus proche Sl-IAA homologue d'un point de vue phylogénétiquement, a mis en évidence un domaine conservé chez ces deux protéines de type YxGLS présent dans la partie N-amino terminale avant le domaine I. La présence de ce domaine dans deux Aux/IAA distincts ayant une fonction en tant que régulateur du développement du fruit ou de son initiation suggère un lien entre ce domaine et la fonction de la protéine. En outre des analyses par double hybride

ont permis de montrer que Sl-IAA27 est capable d'interagir avec tous les ARF activateurs testés et avec trois des protéines TOPLESS identifiées chez la tomate.

I Introduction

The understanding of auxin control of plant development was in part mediated by the analysis of *Aux/IAA* mutants in Arabidopsis. Indeed as described in chapter I Aux/IAA proteins are key components of auxin signaling in response to the auxin perception by the complex SCF^{TIR1}. Analysis of dominant or semi-dominant *Aux/IAA* gain-of-function Arabidopsis mutants notably revealed numerous phenotypes including altered tropisms, apical dominance, organ patterning and structure (Rouse et al. 1998; Tian and Reed 1999; Nagpal et al. 2000; Rogg et al. 2001; Fukaki et al. 2002; Tatematsu et al. 2004; Overvoorde et al. 2005; Uehara et al. 2008). Gain-of-function Arabidopsis mutants result from mutation in the highly conserved Aux/IAA domain II which leads to the stabilization of Aux/IAA proteins (Uehara et al. 2008). However, because Aux/IAA protein stabilization may not mimic regulatory events actually occurring in wild-type plants, the study of loss-of-function mutants would allow better determination of Aux/IAA physiological significance. Unfortunately, in Arabidopsis, the null mutants failed to show visible phenotypes, probably as a result of extensive functional redundancy (Overvoorde et al. 2005). In contrast, the down-regulation of *Sl-IAA9* results in pleiotropic phenotypes. Transgenic plants displayed an alteration of leaf morphogenesis with the formation of simple leaves but also fruit set prior to fertilization leading to the formation of parthenocarpic fruits (Wang.H et al. 2005). This analysis showed that the Sl-IAA9 protein is a pivotal mediator of auxin regulation of the processes of fruit set and leaf formation. Moreover, the tomato appeared to be a complementary suitable plant model in addition to Arabidopsis to analyze Aux/IAA physiological significance by the study of *Aux/IAA* loss-of-function plants. In consequence, the strategy chosen by the laboratory was to generate transgenic tomato plants for all *Aux/IAA* identified. In following years, phenotypic analysis of *Sl-IAA3* down-regulated plants revealed that this gene could be a molecular link between

auxin and ethylene pathways in tomato. Indeed plants displayed both auxin and ethylene-related developmental defects, including reduced apical dominance, reduced auxin response and exaggerated apical hook in etiolated seedlings (Chaabouni et al. 2009a, Chaabouni et al. 2009b). More recently, it has been shown that *Sl-IAA15* down-regulated lines display lower trichome number, reduced apical dominance with associated modified pattern of axillary shoot development, increased LR formation and decreased fruit set. Moreover, the leaves of *Sl-IAA15* inhibited plants are dark green and thick, with larger pavement cells, longer palisade cells and larger intercellular space of spongy mesophyll cells (Deng et al. 2012). Overall, three distinct *Aux/IAA* down-regulating plants displayed pleiotropic phenotypes revealing that members of the *Aux/IAA* gene family perform both overlapping and specific functions in tomato.

At the beginning of my PhD, various transgenic lines were generated either over-expressing or under-expressing one Aux/IAA. These plants were generated thanks to the transformation team working in the laboratory which selected homozygous lines after plant transformation. Therefore in addition to previous characterized *Aux/IAA* genes, transgenic plants were available for 13 *Sl-IAAs*. No transgenic plants were generated for the seven *Aux/IAAs* recently identifies thanks to the genome sequencing *(Sl-IAA21, Sl-IAA22, Sl-IAA23, Sl-IAA32, Sl-IAA33, Sl-IAA35* and *Sl-IAA36*) (Table 1). Phenotypic analyses of numerous *Aux/IAA* over-expressing plants were performed before the beginning of my PhD and revealed auxin-relative phenotypes such as dwarf plants or a loss of apical dominance but no phenotype regarding fruit development. Therefore, first phenotypic analyses performed during my PhD were focused on none yet characterized under-expressing *Sl-IAA* plants. Analyses were realized on *Sl-IAA16*, *Sl-IAA17*, *Sl-IAA27* and *Sl-IAA29* RNAi plants focusing on flower morphogenesis, fruit set and ripening (shape, color, firmness, sugar content). First study of *Sl-*

IAA29 RNAi showed an alteration of flower and fruit morphogenesis with the formation of fasciated fruits. Nevertheless this phenotype was no more observed in following analysis. It was previously shown that fasciated phenotype is associated with a modification of environmental conditions and notably to cold temperature (Asahira et al. 1982), suggesting that *Sl-IAA29* RNAi phenotype firstly seen could be due to increase sensitivity to abiotic stresses. Regarding *Sl-IAA16* RNAi plants no altered development was observed for flower and fruit. At the opposite fruits phenotypes were observed in both *Sl-IAA17* and *Sl-IAA27* RNAi plants, with notably a modification of fruit shape and seed number. Due to the fact that phenotypes of *Sl-IAA27* RNAi plants were more significant my PhD work was then focused on the understanding of *Sl-IAA27* function. Results obtained are presented at the following in the form of an article which has been submitted to the Plant and Cell Physiology journal.

Tableau 1: Sl-IAA transgenic lines generated. NG=not generated

Gene name	Over-expressing lines		RNAi or Antisens lines	
	Number of homozygous lines/generated lines	Phenotypic analyses	Number of homozygous lines/generated lines	Phenotypic analyses
Sl-IAA1	1/13	Partially done	NG	
Sl-IAA2	NG		NG	
Sl-IAA3	**Chaabouni et al.** J Exp Bot. 2009;60(4):1349-62			
Sl-IAA4	3/10	Partially done	NG	
Sl-IAA7	5/22	Partially done	NG	
Sl-IAA8	6/24	Partially done	NG	
Sl-IAA9	**Wang et al.** Plant Cell. 2005 Oct;17(10):2676-92			
Sl-IAA11	NG		NG	
Sl-IAA12	NG		4/25	Partially done
Sl-IAA13	5/14	Partially done	NG	
Sl-IAA14	3/17	Partially done	NG	
Sl-IAA15	**Deng et al.** New Phytol. 2012 Apr;194(2):379-90			
Sl-IAA16	**5/27**	**Partially done**	**4/10**	**Done**
Sl-IAA17	**3/10**	**Partially done**	**3/7**	**Done**
Sl-IAA19	3/14	Partially done	NG	
Sl-IAA21	NG		NG	
Sl-IAA22	NG		NG	
Sl-IAA23	NG		NG	
Sl-IAA26	4/7	Partially done	NG	
Sl-IAA27	**5/25**	**Done**	**11/21**	**Done**
Sl-IAA29	**5/15**	**Done**	**9/25**	**Done**
Sl-IAA32	NG		NG	
Sl-IAA33	NG		NG	
Sl-IAA35	NG		NG	
Sl-IAA36	NG		NG	

II Publication

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Phenotypes Associated with Down-Regulation of *Sl-IAA27* Support Functional Diversity Among Aux/IAA Family Members in Tomato

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The phytohormone auxin is known to regulate several aspects of plant development, and Aux/IAA transcription factors play a pivotal role in auxin signaling. To extend our understanding of the multiple functions of Aux/IAAs further, the present study describes the functional characterization of *Sl-IAA27*, a member of the tomato *Aux/IAA* gene family. *Sl-IAA27* displays a distinct behavior compared with most Aux/IAA genes regarding the regulation of its expression by auxin, and the *Sl-IAA27*-encoded protein harbors a unique motif of unknown function also present in Sl-IAA9 and remarkably conserved in monocot and dicot species. Tomato transgenic plants underexpressing the *Sl-IAA27* gene revealed multiple phenotypes related to vegetative and reproductive growth. Silencing of *Sl-IAA27* results in higher auxin sensitivity, altered root development and reduced Chl content in leaves. Both ovule and pollen display a dramatic loss of fertility in *Sl-IAA27* down-regulated lines, and the internal anatomy of the flower and the fruit are modified, with an enlarged placenta in smaller fruits. In line with the reduced Chl content in *Sl-IAA27* RNA interference (RNAi) leaves, genes involved in Chl synthesis display lower expression at the level of transcript accumulation. Even though *Sl-IAA27* is closely related to *Sl-IAA9* in terms of sequence homology and the encoded proteins share common structural features, the data indicate that the two genes regulate tomato fruit initiation and development in a distinct manner.

Keywords: Auxin • Aux/IAA • Chl • Fruit • Tomato.

Abbreviations: AFB, auxin receptor F-box; ARF, auxin response factor; Aux/IAA, auxin/IAA; AuxRE, auxin responsive *cis*-element; CaMV, *Cauliflower mosaic virus*; Chl, chlorophyll; EAR, ethylene-responsive element-binding factor-associated amphiphilic repression; GFP, green fluorescent protein; GH3, Gretchen Hagen 3, GUS, β-glucuronidase; MS medium, Murashige and Skoog medium; NAA, naphthalene-1-acetic acid; NLS, nuclear localization signal; NPA, *N*-1-naphthylphtalamic acid; qRT–PCR, quantitative reverse transcription–PCR; RNAi, RNA interference; SAUR, small auxin up RNA; SCF, SKP1-Cullin-F-box; SGN, Solanaceae Genomics Network; Sl-IAA, *Solanum lycopersicum* auxin/IAA; TIR1, transport inhibitor response1; TPL, topless; YFP, yellow fluorescent protein.

Introduction

The phytohormone auxin (IAA) plays a determinant role in plant development by notably controlling cell division, expansion and differentiation and by regulating organ initiation, embryogenesis and root development (Vanneste and Friml 2009). Auxin is also fundamental for successful fertilization of the flower and for fruit initiation and subsequent growth (Gillaspy et al. 1993, Vivian-Smith and Koltunow 1999, Carmi et al. 2003, Rotino et al. 2005). Components of the auxin signaling pathway have been shown to be involved in repressing fruit initiation until the fertilization cue (Vivian-Smith et al. 2001, Wang et al. 2005, Goetz et al. 2006, Pandolfini et al. 2007). Pioneering studies on Arabidopsis have identified different components of auxin signaling, among which the *Aux/IAA* family of transcriptional regulators (Abel et al. 1995). *Aux/IAA* genes constitute one of the three major groups of primary auxin-responsive genes which also include *Small Auxin Up RNA* (*SAUR*) and *Gretchen Hagen 3* (*GH3*) (Theologis et al. 1985, Oeller et al. 1993). *Aux/IAA* genes encode short-lived proteins that typically share four conserved domains (Reed 2001) and display the ability to function as transcriptional repressors due to a conserved leucine motif (LxLxLx) located in domain I (Tiwari et al. 2004) similar to the so-called EAR (ethylene-responsive element binding factor-associated amphiphilic repression) repression domain (Kagale et al. 2010, Kagale and Rozwadowski 2010, Kagale and Rozwadowski 2011). It is well acknowledged now that *Aux/IAA* genes have a dual activity, one as a transcriptional regulator and another as

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a component of the auxin receptor complex. That is, in the absence of auxin, Aux/IAAs can bind ARFs (auxin response factors) through domains III and IV present in the C-terminal part of both proteins and recruit the TOPLESS (TPL) co-repressors, thus preventing ARFs from activating the transcription of their target genes (Guilfoyle and Hagen 2007, Szemenyei et al. 2008).

The presence of auxin promotes the association of Aux/IAAs with the SKP1-Cullin-F-box (SCF) complex through binding to the auxin transport inhibitor response1 (TIR1) or to its paralogs AUXIN RECEPTOR F-BOX (AFB) proteins. The SCF complex targets Aux/IAAs to the proteasome, leading to their rapid degradation (Dharmasiri et al. 2005a, Dharmasiri et al. 2005b, Kepinski and Leyser 2005, Leyser 2006, Tan et al. 2007, Chapman and Estelle 2009). The degradation of Aux/IAAs results in the release of ARFs which can then activate the transcription of target genes via binding to the auxin responsive elements (AuxREs) present in the promoter regions of auxin-regulated genes (Hagen et al. 1991, Ulmasov et al. 1997, Hagen and Guilfoyle 2002). In Arabidopsis, the unraveling of Aux/IAA functions in planta was almost exclusively achieved from the characterization of gain-of-function mutants. Indeed, phenotypes associated with loss-of-function mutations show an important functional redundancy among Aux/IAA family members (Rouse et al. 1998, Tian and Reed 1999, Nagpal et al. 2000, Rogg et al. 2001, Fukaki et al. 2002, Tatematsu et al. 2004, Overvoorde et al. 2005, Uehara et al. 2008). In contrast, down-regulation of *Aux/IAA* genes in tomato proved to be, so far, quite efficient in revealing the physiological significance of a number of Aux/IAAs (Wang et al. 2005, Chaabouni et al. 2009a, Deng et al. 2012). Therefore, this Solanaceae reference species is a suitable system for further deciphering the specific roles of various members of the *Aux/IAA* gene family made up of 25 genes (Audran-Delalande et al. 2012). Using a reverse genetic approach, aiming to down-regulate the expression of *Sl-IAA9* in tomato, revealed the specific role of this Aux/IAA as a switch of the fruit set process and regulator of leaf morphogenesis (Wang et al. 2005). Likewise, the underexpression of the tomato *Sl-IAA3* gene resulted in both auxin- and ethylene-related developmental defects including reduced apical dominance and an exaggerated apical hook in dark-grown seedlings in the absence of ethylene, thus supporting the hypothesis that *Sl-IAA3* may act as a molecular link between ethylene and auxin signaling (Chaabouni et al. 2009a, Chaabouni et al. 2009b). More recently, it was reported that *Sl-IAA15* down-regulated lines display a lower trichome number, reduced apical dominance, increased lateral root formation and dark green leaves (Deng et al. 2012). Taken together, these phenotypes uncover specialized roles for Aux/IAAs in plant developmental processes, clearly indicating that members of the *Aux/IAA* gene family in tomato perform both overlapping and specific functions.

To gain further insight into the physiological significance and diversity of roles associated with Aux/IAAs in tomato, the present study addresses the function of the *Sl-IAA27* gene in planta. The expression pattern of *Sl-IAA27* displays an atypical auxin regulation compared with classical *Aux/IAA* genes, and the down-regulation of this *Aux/IAA* gene leads to Chl underaccumulation in the leaves and to altered fruit growth.

Results

Sl-IAA27 is a canonical *Aux/IAA* gene closely related to *Sl-IAA9*

Aux/IAA genes belong to a large family found in many species and comprises 25 members in tomato (Audran-Delalande et al. 2012). Sl-IAA27 is closely related to Sl-IAA9 and Sl-IAA8, these three proteins forming a distinct clade, named clade B, in the tomato Aux/IAA protein family (Audran-Delalande et al. 2012). To shed more light on this clade B, members were compared with those from the same clade in potato (SGN database: Solanaceae Genomics Network, http://www.sgn.cornell), poplar (Kalluri et al. 2007), rice (Jain et al. 2006) (DRTB database http://drtf.cbi.pku.edu.cn/index.php) and maize (Wang et al. 2010b). Alignment of amino acid sequences of clade B Aux/IAAs revealed the presence in IAA9 and IAA27 of a conserved motif (YxGLS) before domain I in all monocot and dicot species investigated. Notably, the YxGLS motif was also found in Arabidopsis At-IAA8 while it is absent in IAA8 proteins from all other species (**Fig. 1A**). Because this motif is not found in any other Aux/IAA proteins, it provides a specific signature of clade B.

The *Sl-IAA27* coding sequence is 837 bp long and made up of five exons like its closest Arabidopsis homolog (**Fig. 1B**). The derived protein contains 278 amino acids. At the nucleotide and amino acid sequence levels, Sl-IAA27 displays 67 and 52% identity, respectively, with its putative Arabidopsis ortholog At-IAA27. The four conserved domains (I–IV) characteristic of the Aux/IAA family (Abel et al. 1995) are present in Sl-IAA27 protein as well as putative nuclear localization signals (NLSs) with both the bipartite structure of a conserved basic doublet KR between domains I and II and basic amino acids in domain II, and the SV40-type NLS located in domain IV (**Fig. 1B**). The subcellular localization of the Sl-IAA27 protein was assessed by transient expression assay in tobacco protoplasts using a translational fusion between Sl-IAA27 and yellow fluorescent protein (YFP) under the control of the 35S promoter of *Cauliflower mosaic virus* (CaMV). Microscopy analysis showed that in contrast to control protoplasts transformed with YFP alone which displayed fluorescence throughout the cell, the YFP–Sl-IAA27 fusion was only localized in the nucleus (**Fig. 2A**). These data shows that Sl-IAA27 was able to direct the fusion protein to the nucleus, consistent with its putative transcriptional regulatory function. To gain insight into the spatial pattern of expression of the *Sl-IAA27* gene, accumulation of its transcript was assessed in different plant tissues and organs by quantitative reverse transcription–PCR (qRT-PCR) and by analysis of expression of the β-glucuronidase (GUS) reporter gene driven by the *Sl-IAA27* promoter. These data revealed that

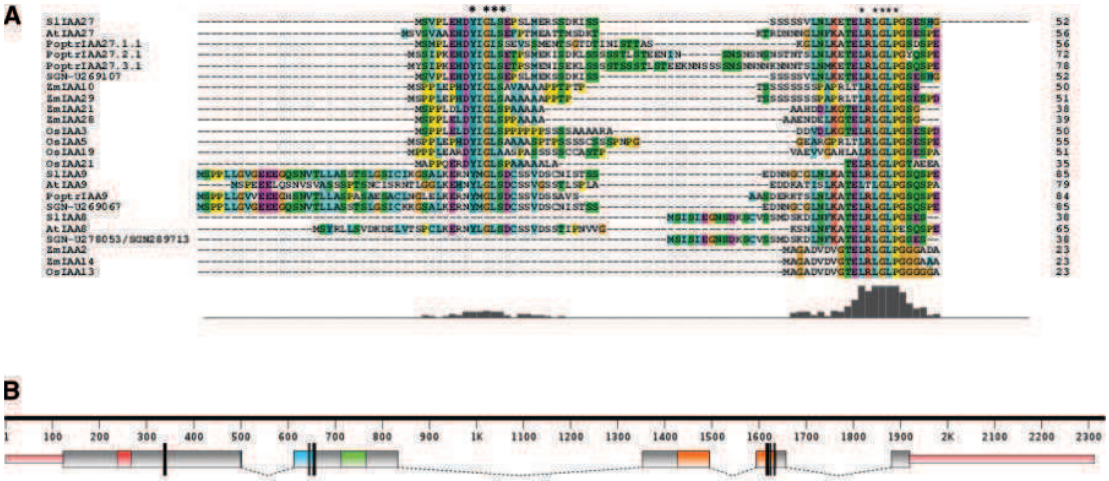

Fig. 1 Structural features of the *Sl-IAA27* gene and derived protein. (A) Multiple sequence alignment of the N-terminal part of Aux/IAA proteins belonging to clade B from tomato, Arabidopsis, maize, rice, potato and poplar obtained with ClustalX and manual correction. Conserved amino acid residues within domain I are shown by stars, as are the conserved YxGLs motif located upstream of domain I and found only in clade B Aux/IAA members. The amino acid position is given on the right of each sequence. (B) Structure of the *Sl-IAA27* gene designed with fancyGENE software (http://host13.bioinfo3.ifom-ieo-campus.it/fancygene/). Exons are represented by gray boxes, introns by hats, untranslated regions by pink boxes, domain I by a red box, domain II by a blue box, domain III by a green box and domain IV by an amber box. Nuclear localization signals are represented by black vertical straight lines.

Sl-IAA27 was expressed in all organs analyzed, with the lowest expression found in leaves (**Fig. 2B**; **Supplementary Fig. S1**). In the flower, *Sl-IAA27* was expressed in sepals, anthers and in the top of the stamen, but not in petals (**Fig. 2B**; **Supplementary Fig. S1D**). The expression was high in the ovary at the anthesis stage (**Supplementary Fig. S1F**). In mature green fruit, the expression was mainly observed in pericarp, endocarp and columella (**Supplementary Fig. S1G**), whereas it was down-regulated during ripening with strongly reduced expression in red fruits (**Fig. 2B**; **Supplementary Fig. S1G**, **H**). In hypocotyls and roots, *Sl-IAA27* is expressed in vascular tissues (**Supplementary Fig. S1A–C**).

Sl-IAA27 transgenic plants showed altered root development and modified auxin sensitivity

Both RNA interference (RNAi) and overexpression approaches were used to address the physiological significance of Sl-IAA27 protein. Several independent homozygous lines were generated; three *Sl-IAA27*-overexpressing lines and three *Sl-IAA27* RNAi lines were selected for further studies. The *Sl-IAA27*-overexpressing lines were named Sline1, Sline2 and Sline3, and the *Sl-IAA27* RNAi lines were named Rline1, Rline2 and Rline3. Analysis by real time-PCR showed that *Sl-IAA27* is overexpressed in the overexpressing transgenic lines with transcript levels 120 times higher than the wild type in Sline1, and up to 50 times higher in Sline2 and Sline3 (**Supplementary Fig. S2**). In the three RNAi lines, real-time PCR experiments showed substantially lower accumulation of *Sl-IAA27* transcripts compared with the wild type. With less than one-third of the mRNA level displayed in the wild type, Rline1 showed the highest level of repression, whereas Rline2 and Rline3 retained half of the normal mRNA levels (**Fig. 2C**). To check the specificity of the RNAi strategy and rule out any potential interference with other *Aux/IAA* genes, the expression of clade B members *Sl-IAA8* and *Sl-IAA9* was assessed. No reduction in the transcript levels of *Sl-IAA8* and *Sl-IAA9* genes was detected, indicating that the altered physiological processes observed in the transgenic lines are primarily due to the down-regulation of *Sl-IAA27*. Moreover, transcript accumulation of *Sl-IAA9*, the most closely related *Aux/IAA* in terms of sequence homology, was even higher in *Sl-IAA27* RNAi lines than in wild-type plants (**Fig. 2C**).

Primary roots were 23–43% more elongated in *Sl-IAA27* RNAi lines than in wild-type plants, and the increase in root length correlated with the level of transcript reduction in the three lines (**Fig. 3A, B**). In addition, *Sl-IAA27* RNAi transgenic lines displayed increased lateral root formation (**Fig. 3A, C**). In contrast, *Sl-IAA27*-overexpressing lines presented a reduced root length, the main root being up to 50% less elongated than in the wild type and producing no lateral root (**Supplementary Fig. S3A**, **B**).

The altered root growth in the transgenic plants suggested that the modification of *Sl-IAA27* expression may result in altered auxin sensitivity. The physiological auxin response of *Sl-IAA27* RNAi and overexpressing plants was assessed using the auxin transport inhibitor *N*-1-naphthylphtalamic acid (NPA) known to alter the endogenous auxin gradients notably in roots. Lateral root formation was inhibited in both wild-type and RNAi plants. Primary root growth was reduced in wild-type, *Sl-IAA27*-overexpressing and RNAi plants with, however, a much lower reduction in the *Sl-IAA27* RNAi lines (**Fig. 4A, B**;

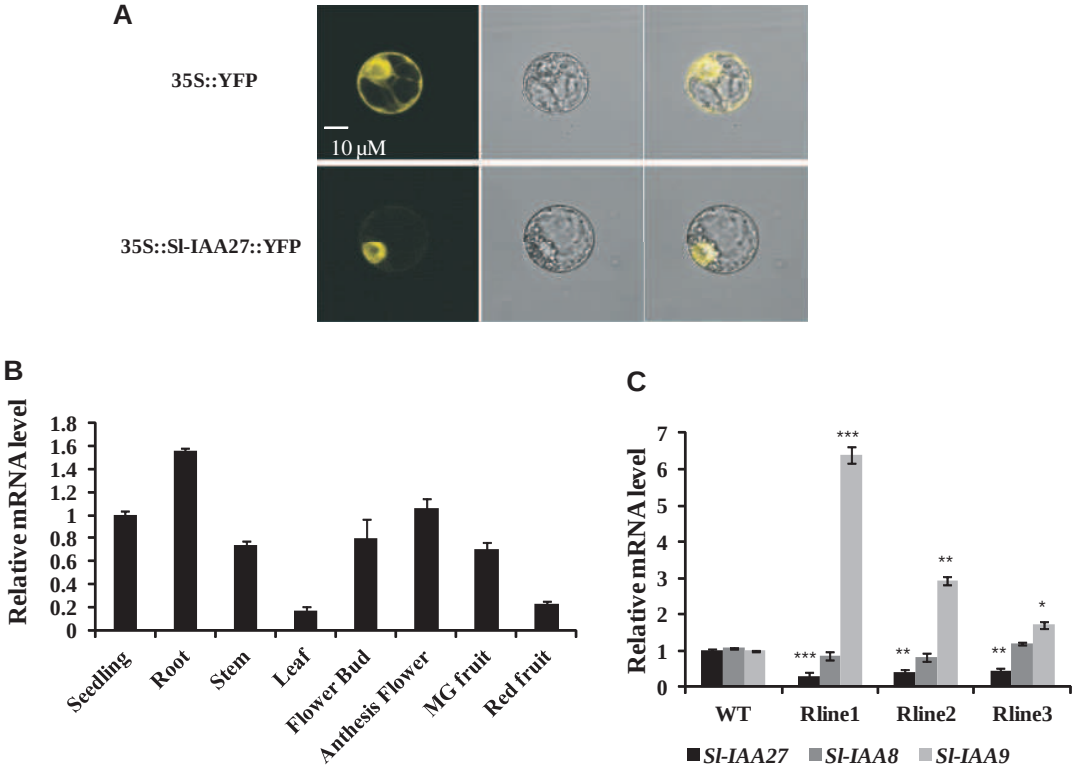

Fig. 2 Subcellular localization of Sl-IAA27 protein and the expression pattern of the *Sl-IAA27* gene at the transcriptional level. (A) Transient transformation in tobacco protoplasts showing the nuclear targeting of the Sl-IAA27 protein fused to YFP and expressed under the control of the 35S promoter. (B) Profiling of *Sl-IAA27* transcript accumulation in various tomato plant tissues monitored by qRT–PCR. Seedlings were used as reference (MG = mature green). (C) Transcript levels of *Sl-IAA27*, *Sl-IAA8* and *Sl-IAA9* genes in three independent *Sl-IAA27* RNAi lines (Rline1, Rline2 and Rline3) assessed in young fruits (10 d after anthesis) by qRT–PCR.

Supplementary Fig. S3C). Due to the difference in NPA sensitivity of *Sl-IAA27* RNAi roots, the auxin sensitivity of *Sl-IAA27* RNAi plants was further explored by determining the auxin dose–response on elongation of hypocotyl segments. **Fig. 4C** shows that independently of the auxin concentration used, the *Sl-IAA27* RNAi hypocotyls were always more elongated than wild-type hypocotyls. After 24 h of auxin treatment, maximum hypocotyl elongation was obtained with a 10^{-5} M NAA (naphthalene-1-acetic acid) concentration in the transgenic lines; while this was within the range of auxin concentrations used in the experiment, it was not reached for the wild type. These data indicate that down-regulation of *Sl-IAA27* confers higher auxin sensitivity to the transgenic plants at the level of hypocotyl elongation.

Because *Aux/IAA* genes have been reported to be up-regulated by auxin (Abel et al. 1994), the auxin responsiveness of the *Sl-IAA27* gene was investigated by qRT–PCR in seedling tissues. The data in **Fig. 4D** clearly indicate that *Sl-IAA27* was down-regulated by auxin treatment. Moreover, using a transient expression experiment in the tobacco protoplast system, the activity of the *Sl-IAA27* promoter fused to green fluorescent protein (GFP) showed no auxin-induced regulation compared with the DR5 synthetic promoter (**Fig. 4E**). In silico analysis of 2,000 bp of the *Sl-IAA27* promoter sequence using PLACE software (http://www.dna.affrc.go.jp/PLACE/index.html) revealed the presence of a variety of conserved motifs, but none of these putative *cis*-elements corresponded to the AuxREs known to drive auxin responsiveness. Strikingly, up to 41 conserved motifs putatively involved in light regulation were found, including a GT1 motif (S000198 GRWAAW), one GT1CORE motif (S000125 GGTTAA) and 17 GATA motifs (S000039 GATA).

Sl-IAA27 RNAi plants showed a lower Chl content

Sl-IAA27 RNAi leaves showed a pale green color more dramatically visible during the first weeks of plant development. In contrast to the green leaves in wild-type plants, transgenic lines presented yellow to light green leaves depending on the level of *Sl-IAA27* down-regulation, with a more marked phenotype in Rline1 (**Fig. 5A**). Chl *a* and *b* content measured by spectrophotometry showed a significant reduction in leaves of the transgenic plants compared with wild-type plants, in line with their light green color (**Fig. 5C**). Notably, the lower level of Chl observed in the RNAi leaves is not related to a diminution of chloroplast number. Indeed, microscopic observation of leaves from the wild type and Rline1 showed that

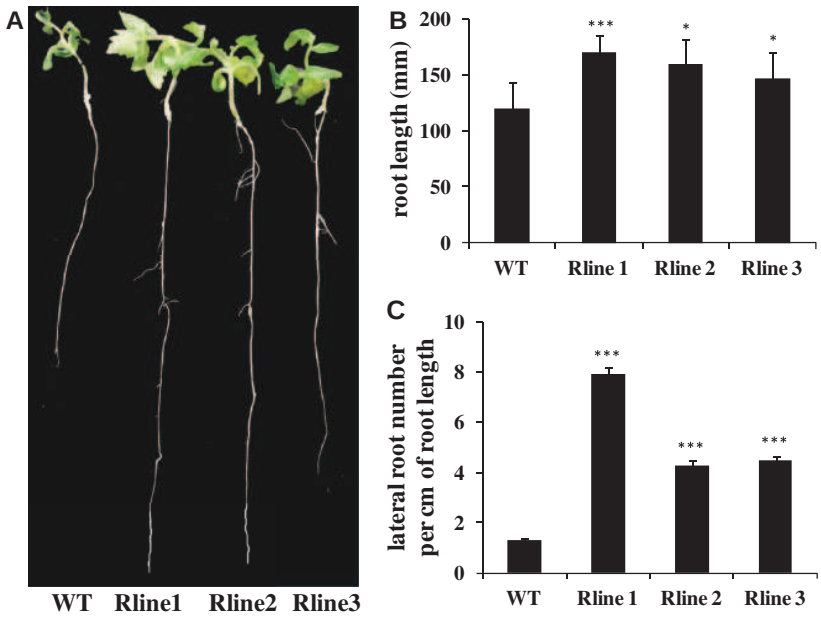

Fig. 3 Altered root growth in *Sl-IAA27* RNAi lines. (A) Root development in the wild type and *Sl-IAA27* RNAi lines assessed in 3-week-old seedlings grown on MS/2 medium. (B) The mean primary root length in the wild type and *Sl-IAA27* down-regulated lines. (C) Lateral root number per cm of root length in the wild type and *Sl-IAA27* RNAi lines. Statistical analysis were realized using the Student's test, $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$. *Sl-IAA27* RNAi lines = Rline1, Rline2 and Rline3.

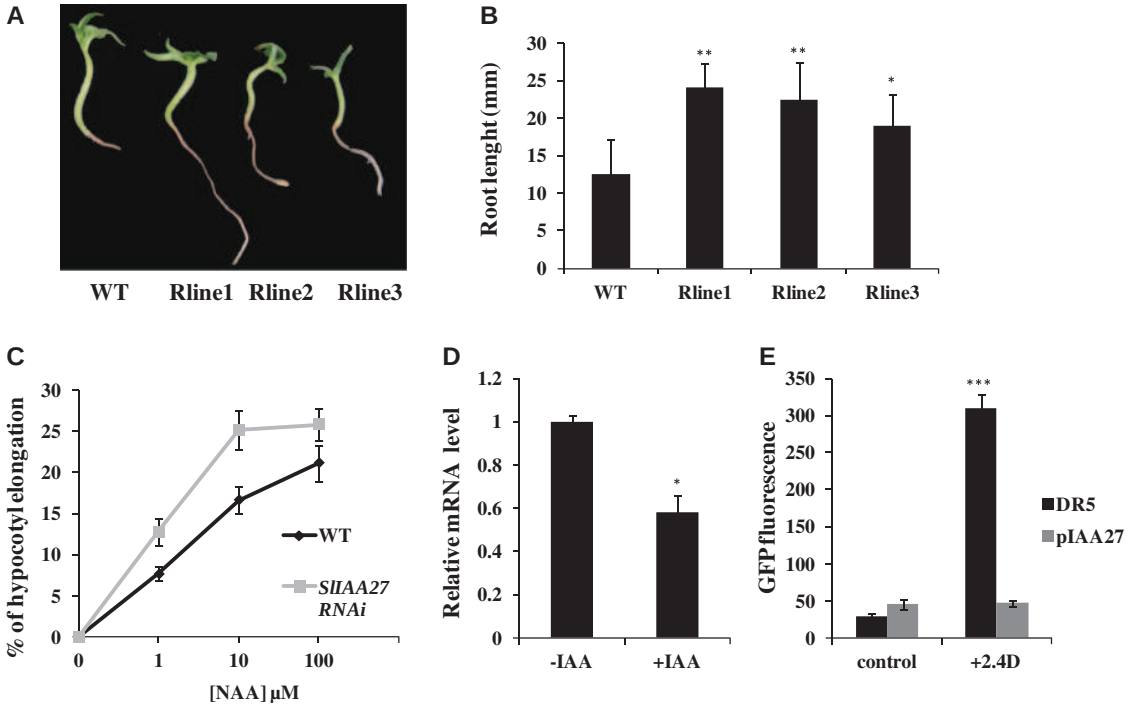

Fig. 4 Altered auxin response in *Sl-IAA27* down-regulated lines. (A) Effect of NPA (5 µM) treatment on root development of 3-week-old wild type (WT) and RNAi tomato lines grown on MS/2 medium. (B) Primary root length of WT and RNAi lines assessed on seedlings treated with NPA. (C) Auxin dose–response of elongation of hypocotyl segments treated with NAA. Hypocotyl segments 9 mm long were cut from 8-day-old seedlings and treated for 24 h with the indicated concentration of NAA. Data represent mean values obtained with three replicates for each of the three *Sl-IAA27* RNAi lines. (D) Auxin regulation of *Sl-IAA27* expression. *Sl-IAA27* transcript accumulation was monitored by qRT–PCR in seedlings treated for 2 h with 20 µM IAA. (E) Auxin responsiveness of the *Sl-IAA27* promoter. GFP fluorescence of protoplasts transformed either with the synthetic promoter DR5 or the *Sl-IAA27* promoter fused to GFP and treated or not with 2,4-D (50 µM). Statistical analyses were realized using the Student's test, $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$. *Sl-IAA27* RNAi lines = Rline1, Rline2 and Rline3.

chloroplast number was not modified but that Chl fluorescence was dramatically reduced as assessed by fluorescence emission (**Fig. 5B**). In *Sl-IAA27*-overexpressing plants, no visible difference was observed in leaf color compared with the wild type. Analysis of Chl *a* and *b* content revealed that the Chl content is not modified by the overexpression of *Sl-IAA27* in leaves (**Supplementary Fig. S4A**).

The lower Chl content in *Sl-IAA27* RNAi leaves was correlated with the down-regulation of genes involved in key steps of Chl biosynthesis. In particular, transcript accumulation of *HEMA1*, *protochlorophyllide reductase a*, *b*, *c* and chelatase subunit *chlh* and *chli* genes displayed significantly lower levels than the wild type, while those corresponding to *aminolevulinate dehydratase* and chelatase subunit *chld* were not affected. Moreover, transcript accumulation of *GUN4*, a positive regulator of Chl biosynthesis (Davison et al. 2005, Peter and Grimm 2009, Adhikari et al. 2011), was also significantly reduced in the transgenic lines (**Fig. 5D**; **Supplementary Fig. S5**, **Supplementary Table S1**). Down-regulation of genes related to Chl biosynthesis was still observed in *Sl-IAA27* RNAi plants in flowers at the anthesis stage and in young fruits (7 d after anthesis) (**Fig. 6A, B**). Analysis of the 2,000 bp promoter sequence of the Chl biosynthesis genes analyzed above showed the presence of AuxREs in all corresponding promoters except for *aminolevulinate dehydratase*, chelatase subunit *chli* and *GUN4*, thus suggesting a putative auxin regulation of most of these genes.

To check whether the down-regulation of Chl biosynthesis-related genes results from the higher sensitivity to auxin of *Sl-IAA27* RNAi plants, the auxin regulation of these genes was assessed at the transcriptional level in wild-type plants. None of these genes showed down-regulation by auxin and, in fact, *HEMA1* and *protochlorophyllide a* genes rather were up-regulated (**Supplementary Fig. S6**). These data suggest that down-regulation of Chl biosynthesis-related genes in the *Sl-IAA27* RNAi lines is not directly induced by higher auxin responsiveness of the plants.

Sl-IAA27 silencing resulted in altered fruit development and reduced fertilization

The effect of *Sl-IAA27* silencing on fruit development was investigated in the three Sl-IAA27 RNAi lines. While the leaves of *Sl-IAA27* RNAi plants display a pale green color compared with the wild type, the fruit color is not modified at any stage of fruit development and ripening (**Supplementary Fig. S7**). However, down-regulation of *Sl-IAA27* resulted in alteration of fruit morphology, with the formation of fruits with a modified shape and reduced size. Indeed, *Sl-IAA27* RNAi fruits displayed a lower fruit width but normal fruit length (**Fig. 6C**). Fruit volume was dramatically reduced in *Sl-IAA27* RNAi lines compared with wild-type plants (**Fig. 6D**). Modification of *Sl-IAA27* RNAi fruit morphology was correlated with altered flower anatomy. Observation of an *Sl-IAA27* RNAi ovary by microscopy

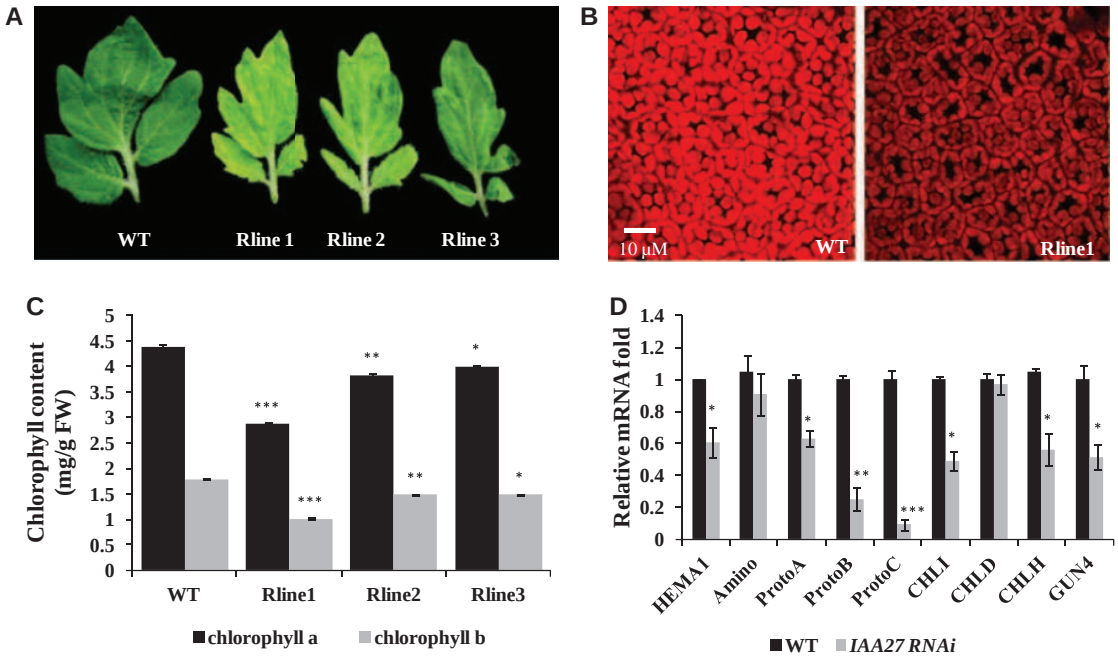

Fig. 5 Alteration of Chl content in *Sl-IAA27* down-regulated lines. (A) Young leaves of *Sl-IAA27* RNAi lines displaying light green color compared with the wild type (WT). (B) Leaf chloroplasts of WT and *Sl-IAA27* Rline1 observed by confocal microscopy. (C) Chl *a* and *b* leaf content (mg g FW^{-1}) in *Sl-IAA27* RNAi and WT lines assessed by spectrophotometry. (D) Transcript accumulation corresponding to genes involved in photosynthesis and Chl biosynthesis monitored by qRT–PCR in leaf tissues of RNAi and WT lines. Statistical analyses were realized using the Student's test, $***P < 0.001$; $**P < 0.01$; $*P < 0.05$. *Sl-IAA27* RNAi lines = Rline1, Rline2 and Rline3.

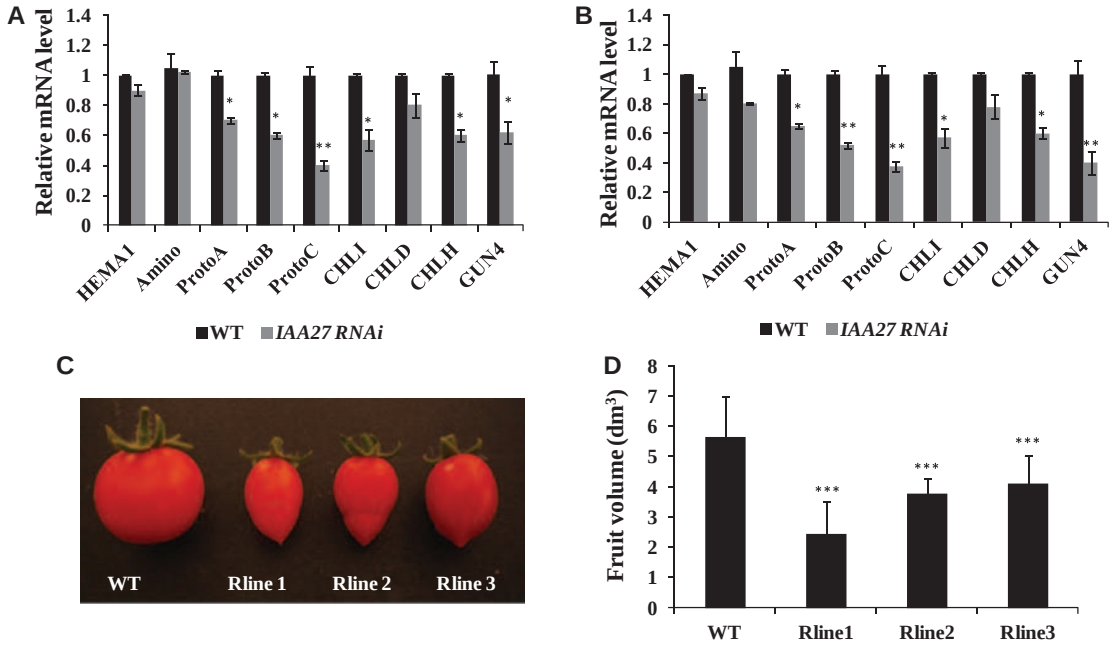

Fig. 6 Altered Chl biosynthesis-related gene regulation and reduced fruit size in *Sl-IAA27* RNAi lines. (A) and (B) Transcript levels of genes involved in photosynthesis and Chl biosynthesis monitored by qRT–PCR in mature flowers at the anthesis stage (A) and in young fruits (10 d after anthesis) (B). (C) Altered fruit shape and volume observed at the full-ripe stage (BK + 10) stage. (D) Fruit volume determined at BK + 10. The data represent mean values obtained with three replicates for each RNAi line. Statistical analyses were realized using the Student's test, $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$. *Sl-IAA27* RNAi lines = Rline1, Rline2 and Rline3.

revealed a modified shape with enlarged placenta and lower number of ovules compared with the wild type (**Fig. 7A**). This altered anatomy structure was maintained in the developed fruits (**Fig. 7B**). Ovule numbers showed a dramatic reduction in Rline1, with an average of 15 ovules per ovary compared with 24 in the wild type (**Fig. 7C**). A decrease in seed number was also observed in *Sl-IAA27* RNAi lines that was, however, more severe than the reduction in ovule number. Indeed, only 27% of Rline1 ovules developed seeds (**Fig. 7C, D**). To check whether in addition to the reduction in number, the ovules also have altered fertility in the transgenic lines, cross-fertilization was performed using transgenic lines as the female recipient and the wild type as the pollen donor. The data presented in the upper part of **Table 1** clearly show that wild-type pollen is unable to increase the seed number, suggesting that ovule fertility is reduced in *Sl-IAA27* RNAi. Moreover, reciprocal crossing using the wild type as the female recipient also revealed a loss of pollen in the transgenic lines (**Table 1**). Overall, these data indicated that both ovules and pollen display reduced fertility in *Sl-IAA27* RNAi. Fruits of *Sl-IAA27*-overexpressing plants displayed no modification in fruit shape or volume, and presented no reduction in seed number (**Supplementary Fig. S4B, C**).

Discussion

Most of our knowledge of the physiological significance of *Aux/IAA* genes comes from the characterization of gain-of-function mutants in Arabidopsis (Rouse et al. 1998, Tian and Reed 1999, Nagpal et al. 2000, Rogg et al. 2001, Fukaki et al. 2002, Tatematsu et al. 2004, Uehara et al. 2008). In Solanaceae species and particularly in tomato, a wide range of phenotypes associated with down-regulation of *Aux/IAA* genes have been reported (Wang et al. 2005, Kloosterman et al. 2006, Chaabouni et al. 2009a, Deng et al. 2012), but the role of most tomato *Aux/IAA* genes still remains to be elucidated. While aiming to illuminate further the multiple functions of *Aux/IAA* genes, the present study more specifically deals with the functional characterization of *Sl-IAA27* for which information regarding the putative function is lacking, in the Solanaceae as well as for its ortholog in Arabidopsis. Silencing of *Sl-IAA27* results in pleiotropic morphological and developmental phenotypes including altered fruit development and Chl accumulation. In these *Sl-IAA27* RNAi plants, both ovule and pollen fertility are reduced, indicating that normal expression of *Sl-IAA27* is required for flower fertility. Furthermore, *Sl-IAA27* down-regulated lines showed a dramatic reduction in ovule formation which, associated with reduced fertility, leads to the production of seedless fruits. Compared with the wild type, the fruit shape and internal fruit structure are also altered, with an enlarged placenta and smaller final fruit size. In *Sl-IAA27*-overexpressing plants, no phenotypes were observed regarding the Chl content in leaves or fruit development, suggesting that even if the *Sl-IAA27* mRNA level is high, its protein level is probably regulated by auxin-mediated degradation, restoring its level to that in the wild type.

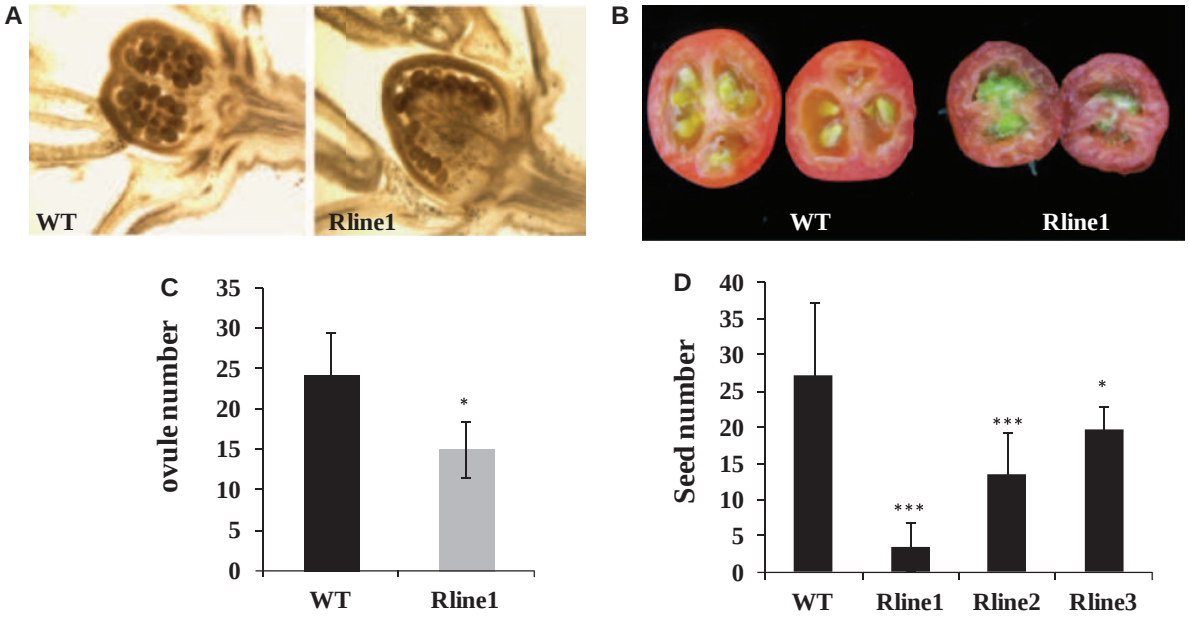

Fig. 7 Altered ovule, seed and fruit development in *Sl-IAA27* down-regulated lines. (A) Ovary shape in mature flowers at the anthesis stage in the wild type (WT) and *Sl-IAA27* Rline1. (B) Fruit shape in WT and *Sl-IAA27* Rline1 displaying smaller size and lower seed content. (C) Average ovule number per flower at the anthesis stage in WT and *Sl-IAA27* Rline1. (D) Seed number in WT and *Sl-IAA27* RNAi mature fruits. Statistical analyses were realized using the Student's test, $***P < 0.001$; $**P < 0.01$; $*P < 0.05$. *Sl-IAA27* RNAi lines = Rline1, Rline2 and Rline3.

Table 1 Fruit set and seed number in cross-pollinated flowers

Female recipient	Pollen donor	Fruit set/crossed flowers	Fruit set (%)	Average seed number per fruit
Rline1	WT	6/30	20	9
Rline2	WT	11/30	37	12
Rline3	WT	11/30	37	21
WT	Rline1	12/30	40	7
WT	Rline2	14/30	47	14
WT	Rline3	13/30	43	18
WT	WT	28/30	93	28

Percentage of fruit set and seed number determined following cross-fertilization of emasculated flowers [wild type (WT) and RNAi lines] with pollen from WT or *Sl-IAA27* down-regulated lines (Rline1, Rline2 and Rline3).

Previous studies showed that down-regulation of either *Sl-IAA3*, *Sl-IAA9* or *Sl-IAA15* results in modified auxin sensitivity associated with altered leaf structure and modified plant architecture (Wang et al. 2005, Chaabouni et al. 2009a, Deng et al. 2012). In contrast, the higher auxin sensitivity of *Sl-IAA27* down-regulated plants did not result in visible alteration of the plant architecture. The transgenic lines display altered primary root growth and lateral root formation. Interestingly, overexpressing and RNAi plants displayed opposite phenotypes, with reduced primary root growth and no lateral root formation in overexpressing lines and increased primary root length with more lateral roots in RNAi lines. This suggests that in roots of overexpressing plants, auxin-dependent degradation of Sl-IAA27 is probably not able to restore protein levels similar to the wild type.

In the presence of the auxin transport inhibitor NPA, *Sl-IAA27* RNAi roots displayed no lateral roots, suggesting that NPA is still able to block transverse divisions as in the wild type (Casimiro et al. 2001). Nevertheless, though root elongation was reduced upon NPA treatment in *Sl-IAA27* RNAi lines, the root length remains more important than in the wild type subjected to the same treatment. Root elongation is controlled by a specific auxin concentration balance, and auxin is known to play a predominant role in root development mainly through controlling cell division (Friml et al. 2002, Ullah et al. 2003, Ding and Friml 2010, Dubrovsky et al. 2011). In Arabidopsis, characterization of several *Aux/IAA* mutants (CRANE/IAA18, SHY2/IAA3, AXR2/IAA7 and SLR/IAA14) revealed the function of these genes in regulating the root architecture (Nagpal et al. 2000, Vanneste et al. 2005, Uehara et al. 2008). NPA application was reported to lead to IAA accumulation in the root tip by blocking auxin basipetal transport in roots (Casimiro et al. 2001). The more elongated primary root in *Sl-IAA27* RNAi plants upon NPA treatment may result from the difference either in auxin concentration or in auxin sensitivity of transgenic lines compared with the wild type. Analysis of the auxin dose–response of hypocotyl elongation where endogenous auxin was removed showed that transgenic plants were more sensitive to NAA. These data indicate that for a specific auxin concentration, *Sl-IAA27* RNAi plants present higher auxin sensitivity, suggesting that Sl-IAA27 protein acts as a repressor of auxin responses. In line with this hypothesis,

previous data showed that Sl-IAA27 represses the auxin-dependent transcription of both synthetic and native auxin-responsive promoters (Audran-Delalande et al. 2012).

In contrast to classical *Aux/IAA* genes characterized by a rapid induction of their expression in response to auxin (Walker and Key 1982, Theologis et al. 1985), *Sl-IAA27* expression is down-regulated by exogenous auxin treatment. *At-IAA27* and *Sb-IAA21*, the *Sl-IAA27* homologs from Arabidopsis and Sorghum, respectively, are also down-regulated by auxin (Paponov et al. 2008, Wang et al. 2010a), suggesting that this feature might be conserved across plant species. Previous analysis of the auxin regulation of tomato *Aux/IAA* genes with short-term treatment has shown that *Sl-IAA8* is also down-regulated by auxin and that *Sl-IAA9* expression is not significantly responsive to auxin (Audran-Delalande et al. 2012). These data indicate that members of clade B of the *Aux/IAA* gene family (*Sl-IAA8*, *Sl-IAA9* and *Sl-IAA27*) behave differently from other members regarding their regulation by auxin. Recently, phylogenetic analysis of the tomato Aux/IAA family revealed that Sl-IAA9 is closely related to Sl-IAA27 (Audran-Delalande et al. 2012), raising the hypothesis that protein structure similarity may result in functional redundancy. Beside domains I–IV which are characteristic of the Aux/IAA family (Abel et al. 1995), our study identified another conserved motif (YxGLS) located before domain I in the N-terminal part. It is noteworthy that this motif, present in both Sl-IAA27 and Sl-IAA9, is not found in any other Sl-IAA and is remarkably conserved in monocot and dicot species, including Arabidopsis, potato, poplar, maize and rice. Whether the conserved YxGLS motif determines some of the functional roles of Sl-IAA27 and Sl-IAA9 remains to be elucidated in planta. Despite their common structural features, the two genes seem to have distinct functions in plant developmental processes, as indicated by the contrasting phenotypes resulting from the down-regulation of *Sl-IAA27* and *Sl-IAA9*. Indeed, down-regulation of *Sl-IAA9* leads to altered leaf morphogenesis (Wang et al. 2005) while such a phenotype is absent in *Sl-IAA27*-underexpressing lines. Also, down-regulation of *Sl-IAA27* results in lower fertility of both ovule and pollen, while reduced expression of *Sl-IAA9* did not affect this (Wang et al. 2005). Moreover, in contrast to *Sl-IAA9* down-regulated plants where fruits display no morphological or size alteration, *Sl-IAA27* RNAi fruits present a reduced size and altered shape. Taken together, these data suggest that normal expression of *Sl-IAA27* is required for the fertilization process and for fruit development, whereas *Sl-IAA9* is essential for triggering fruit initiation (Wang et al. 2005). Even though both *Sl-IAA27* and *Sl-IAA9* down-regulated lines yield seedless fruit, two different mechanisms seem to underlie this phenotype. This is further supported by the observed up-regulation of *Sl-IAA9* expression in *Sl-IAA27* down-regulated plants shown in **Fig. 2C**. Nevertheless, our data suggest a link between the two genes while revealing that the expression of *Sl-IAA9* is regulated by *Sl-IAA27*, either directly or indirectly.

Even though the level of *Sl-IAA27* expression is lowest in wild-type leaves, the phenotypes displayed by the RNAi lines indicate that a threshold level of *Sl-IAA27* expression is necessary to promote Chl biosynthesis in leaves. On the other hand, the overexpression of *Sl-IAA27* does not result in any increase in Chl content in leaves, suggesting that the basal Sl-IAA27 protein level in the wild type is sufficient to sustain maximum activity of Chl biosynthesis genes. This may explain the lack of effect on Chl content in the overexpressing lines. The reduced Chl content of *Sl-IAA27* RNAi plants correlates with the down-regulation of many genes involved in Chl biosynthesis in leaves. Moreover, *Sl-IAA27* seems to regulate at the transcriptional level the expression of the *GUN4* gene which encodes a transcription factor known to activate the Chl biosynthesis pathway. The presence of AuxREs in the promoter of most of these genes suggests that they may potentially undergo direct regulation by auxin signaling. In wild-type plants, the expression of *HEMA1* and *ProtoA* genes is up-regulated upon auxin treatment, while in *Sl-IAA27* RNAi plants the expression of these two genes is down-regulated. To explain this apparent contradiction, it is possible to consider that these two genes are under competitive regulation by activator and repressor ARFs. Taking into account that auxin accumulation is known to lead to the degradation of the majority of Aux/IAA proteins which frees both activator and repressor ARFs (Dharmasiri et al. 2005a, Dharmasiri et al. 2005b, Kepinski and Leyser 2005, Leyser 2006, Tan et al. 2007, Chapman and Estelle 2009), it can be hypothesized that in normal situations the expression of genes related to Chl biosynthesis is down-regulated by one or more repressor ARFs that are trapped by Sl-IAA27 protein. These repressor ARFs are released in the absence of Sl-IAA27 in RNAi plants or upon auxin treatment. On the other hand, the release of activator ARFs in the presence of auxin may result in the up-regulation of *HEMA1* and *ProtoA* genes observed in wild-type plants. Taken together, the data suggest a complex and finely tuned mechanism of regulation of auxin responses involving Aux/IAAs and ARFs.

The smaller size of the *Sl-IAA27* down-regulated fruits may result from the reduced photosynthetic activity in the leaves. This hypothesis is supported by the prevailing opinion that fruit growth is predominantly supported by photoassimilate supply from source tissues (Lytovchenko et al. 2011). In keeping with the prominent role of photosynthetic activity in the leaves, down-regulation of Chl biosynthesis in a fruit-specific manner indicated that photosynthesis was essential for seed set but did not affect fruit growth (Lytovchenko et al. 2011). It is important to mention, however, that cells in developing fruit contain photosynthetically active chloroplasts and express both nuclear-encoded and plastid-encoded genes for photosynthetic proteins (Piechulla et al. 1987). It has been reported that during early fruit development, photosynthesis itself may provide a significant contribution to sustain the growth of the organ (Obiadalla-Ali et al. 2004). More recently, global transcriptomic profiling of fruit set and early fruit development also revealed a strong activation of photosynthesis-related genes

(Wang et al. 2009). Interestingly, our present data show that the expression of a number of Chl biosynthesis genes is down-regulated during the initial phase of fruit development in *Sl-IAA27* RNAi lines, which may impact final fruit size. In line with this hypothesis, previous studies indicated that the induction of genes related to photosynthesis and chloroplast biogenesis in tomato pericarp cells is positively correlated with fruit size (Kolotilin et al. 2007). While taking these data together supports the idea that both leaf and fruit photosynthetic activities may contribute to sustain fruit growth, the relative contribution of the two source tissues to the needs of the fruit in terms of photoassimilate supply remains unclear.

Materials and Methods

Plant materials and growth conditions

Tomato plants (*Solanum lycopersicum* cv. MicroTom) were grown under standard greenhouse conditions. The conditions were as follows: 14 h day/10 h night cycle, 25/20°C day/night temperature, 80% relative humidity, 250 µmol m^{-2} s^{-1} light intensity (intense luminosity). Tomato seeds were first surface sterilized for 5 min in bleach and water, rinsed five times in sterile water and sown in recipient Magenta vessels containing 50 ml of 50% Murashige and Skoog (MS) culture medium and 0.8% (w/v) agar, pH 5.9.

Plant transformation

To generate *Sl-IAA27* RNAi plants, the forward 5′-atgtctgtaccattag-3′ and reverse 5′-gttctgagtctcatgg-3′ primers were used to amplify the 155 bp *Sl-IAA27* RNAi sequence. This fragment was cloned into the pHellsgate12 vector using Gateway cloning. To generate *Sl-IAA27*-overexpressing plants, the forward 5′-atgtctgtaccattag-3′ and reverse 5′-ctaattttggttcttgc-3′ primers were used to amplify the 837 bp of *Sl-IAA27* coding sequence. This fragment was cloned into the pMDC32 vector using Gateway cloning. To generate *pIAA27*–GUS (the promoter of the *Sl-IAA27* gene fused to the GUS reporter gene) plants, the forward 5′-catacaggagatatggattg-3′ and reverse 5′-tgctcaactttcc-3′ primers were used to amplify the 2,000 bp *SlIAA27* promoter sequence. This fragment was cloned into the pMDC162 expression vector by Gateway cloning. Transgenic plants were generated by *Agrobacterium tumefaciens*-mediated transformation according to Wang et al. (2005). All experiments were carried out using homozygous lines from the F_2 and F_3 generation selected on kanamycin resistance.

Histochemical GUS analysis

For histochemical GUS analysis, tissues of *pIAA27*–GUS lines were put in GUS staining solution (100 mM sodium phosphate buffer, pH 7.2, 10 mM EDTA, 0.1% Triton X-100, 0.3 mg ml^{-1} X-Gluc); a vacuum was made for 15 min twice. Tissues were then incubated in GUS staning solution at 37°C overnight. Samples were then decolorated using several washes of graded ethanol series as indicated by Wang et al. (2005).

Transient expression using a single cell system

For nuclear localization of the Sl-IAA27 protein, the *Sl-IAA27* CDS sequence was cloned by Gateway technology (forward 5′-atgtctgtaccattag-3′ and reverse 5′-gcaagaaccaaaattag-3′ primers) in-frame with YFP into the pEarlyGate104 vector, and expressed under the control of the 35S CaMV promoter. The empty vector pEarleyGate104 was used as control. For the analysis of *Sl-IAA27* promoter regulation by auxin, the 2,000 bp of the *Sl-IAA27* promoter sequence was fused to the GFP reporter gene by Gateway technology, into the pMDC107 vector. Protoplasts were obtained from suspension-cultured tobacco (*Nicotiana tabacum*) BY-2 cells and transfected according to the method described previously (Leclercq et al. 2005). YFP localization by confocal microscopy and GFP measurement by flux cytometry were performed as described previously (Audran-Delalande et al. 2012).

Hormonal treatment

For hypocotyl auxin dose–response (0, 1, 10 and 100 µM NAA) and NPA treatment, experiments were carried out as described by Chaabouni et al. (2009a). Auxin treatment of seedlings was performed as previously described by Audran-Delalande et al. (2012).

RNA isolation and real-time PCR analysis

RNA extraction, cDNA synthesis and qRT–PCR analyses were performed as previously described (Pirrello et al. 2006). The primer sequences are listed in **Supplementary Table S2**.

Determination of Chl content

A 100 mg aliquot of leaves from 3-week-old *Sl-IAA27* RNAi and wild-type plants was weighed and ground with 1 ml of 80% acetone. The liquid obtained was centrifuged for 1 min at 10,000 r.p.m. to remove any remaining solid tissue. Samples were analyzed by spectrophotometry at two wavelengths, 645 and 663 nm, using 80% acetone as the blank. The Chl *a* and *b* content was determined using the following equations: Chl $a = 0.999A_{663} - 0.0989A_{645}$ and Chl $b = -0.328A_{663} + 1.77A_{645}$

Microscopy analysis

For chloroplast observation, air was removed from leaves which were then analyzed with a Leica TCS SP2 confocal laser scanning microscope. Images were obtained with a ×40, 1.25 numerical aperture water-immersion objective. Excitation was at 488 nm and the emitted light was captured at 505–535 nm. For flower observation, flowers at the anthesis stage were fixed in ethanol and included in 5% agarose. Flowers were cut every 80 µm using a vibratome (vibratome Leica VT 100S) and then observed with a Leica DM IRBE inverted microscope.

Phenotypic analysis

The phenotypic analyses were performed on fruits of 10 plants from each transgenic line and from the wild-type plants cultivated in the same conditions. The inflorescence formation occurred at the same time in transgenic plants and in the wild type. Phenotypic analyses were performed on the six first fruits developed on each plant, which were similar with regard to their stage of development in transgenic plants and in the wild type (data not shown). In total, 60 fruits of the transgenic lines and the wild type were studied.

The experiment was done twice. Fruit color (Hue angle) was determined using a Chroma Meter CR-310 Minolta. Cross-pollination between *Sl-IAA27* RNAi and WT flowers was performed as described in Wang et al. (2005).

Sequence data

To identify homologs of tomato clade B, phylogenetic analysis were performed with all Aux/IAA members in maize, rice and poplar genomes, available, respectively, in the Maize GDB genome browser tool (http://gbrowse./maizegdb.org/gbrowse/cgi-bin/gbrowse/maize) and KOME database (http://www.cdna.01.dna.affrc.go.jp/cDNA/), and *Populus trichocarpa* Genome database (http://www.plantgdb.org/PtGDB/). Both BLASTN and TBLASTN search were performed on the whole set of potato unigenes in the SGN database (Solanaceae Genomics Network, http://www.sgn.cornell) using Aux/IAA tomato clade B sequences.

Sequence data for the Arabidopsis genes used in this article can be found in the Arabidopsis Genome Initiative data library under the following accession numbers: *AtIAA8* (AT2G22670), *AtIAA9* (AT5G65670) and *AtIAA27* (AT4G29080).

Sequence data for maize genes used in this article can be found in Maize GDB genome browser tool (http://gbrowse./maizegdb.org/gbrowse/cgi-bin/gbrowse/maize) under the following accession numbers: *ZmIAA2* (GRMZM2G077356_PO1), *ZmIAA10* (GRMZM2G037369_PO1), *ZmIAA14* (GRMZM2G077356_PO1), *ZmIAA21* (GRMZM2G147243_PO2), *ZmIAA28* (GRMZM2G035465_PO3) and *ZmIAA29* (GRMZM2G163848_PO5).

Sequence data for the rice genes used in this article can be found in the KOME database (http://www.cdna.01.dna.affrc.go.jp/cDNA/) under the following accession numbers: *OsIAA3* (AK104654), *OsIAA5* (AK106121), *OsIAA13* (AK059838), *OsIAA19* (AK109363) and *OsIAA21* (AK121989).

Sequence data for the poplar genes used in this article can be found in the *P. trichocarpa* genome database under the following accession numbers: *PoptrIAA27.1.1* (POPTR_0001s18680.1), *PoptrIAA27.2.1* (POPTR_0003s04980.1), *PoptrIAA27.3.1* (POPTR_0006s16640.1) and *PoptrIAA9* (POPTR_0002s10880.1).

Sequence data for the tomato genes used in this article can be found in Genbank/EMBL data libraries under the following accession numbers: *Sl-IAA8* (JN379436), *Sl-IAA9* (JN379437) and *Sl-IAA27* (JN379450).

Supplementary data

Supplementary data are available at PCP online

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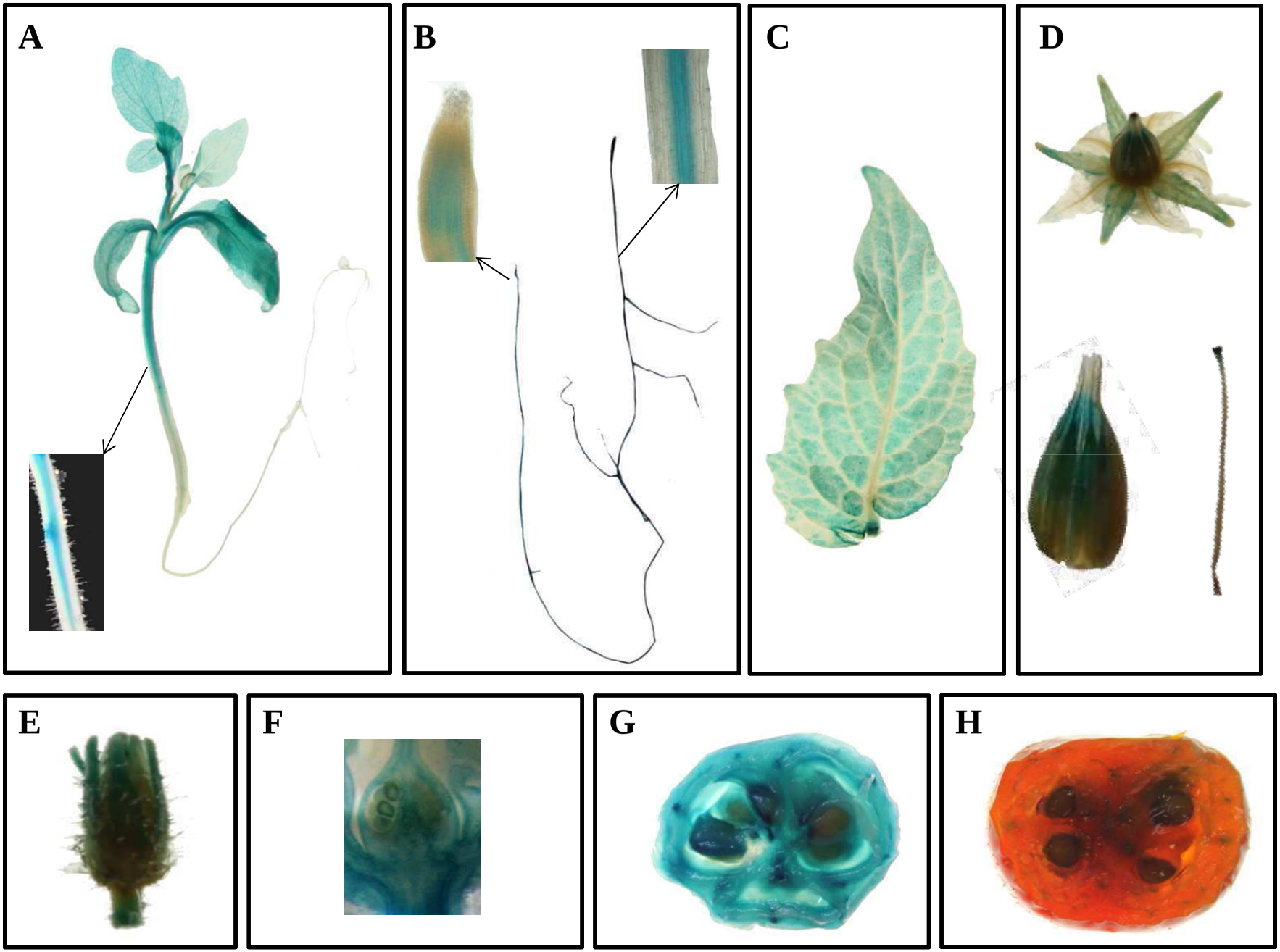
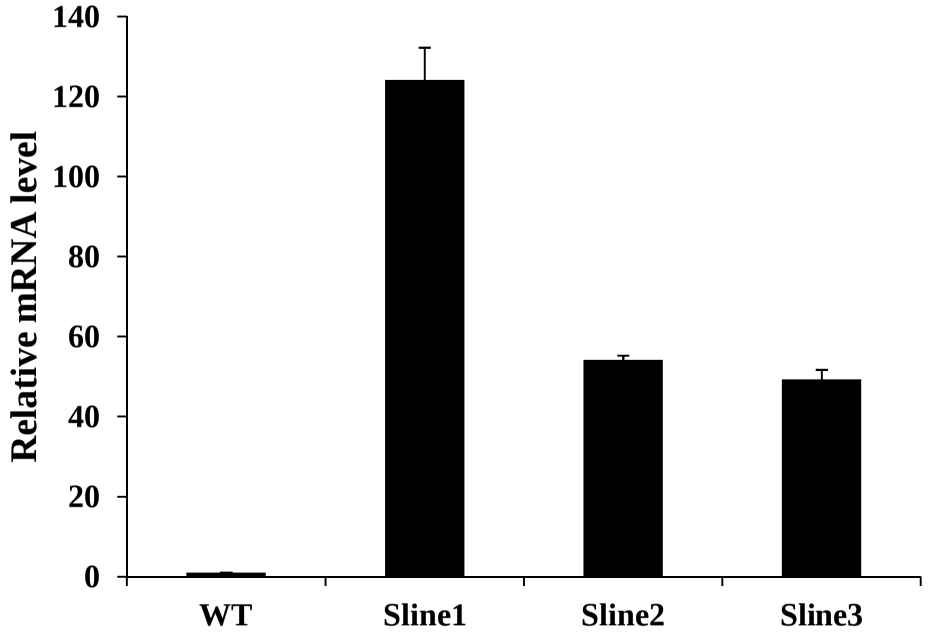
Fig S1



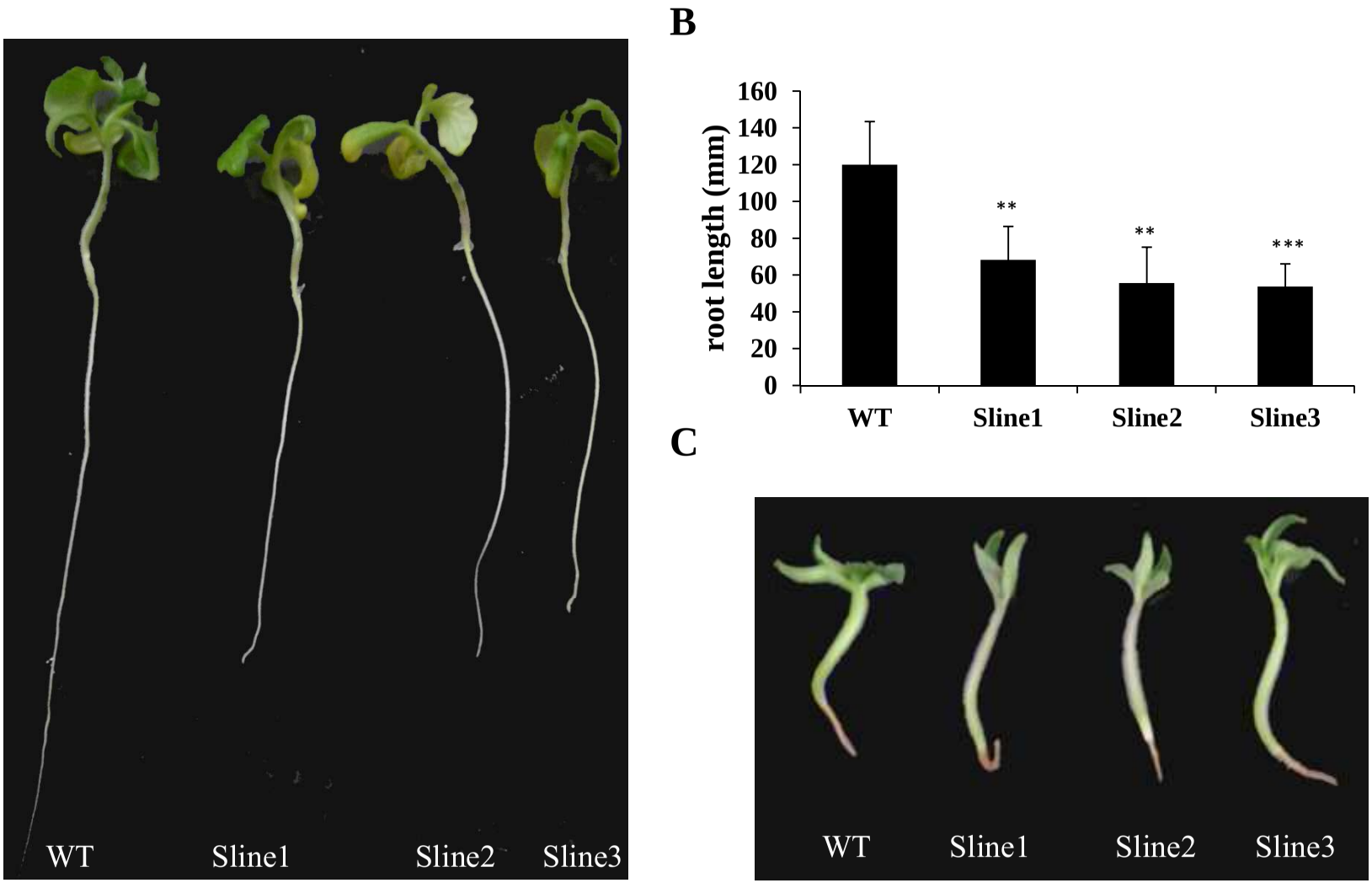
Supplementary Figure S1. *Sl-IAA27* pattern of expression assessed by analysis of GUS reporter gene expression driven by *Sl-IAA27* promoter (*pIAA27*-GUS) (A) seedling with focus on hypocotyl segment (B) root with focus at root apex (C) leaflet (D) flower at anthesis stage, anther and stamen (E) bud (F) ovary of flower at anthesis stage (G) mature green fruit (H) red fruit

Fig S2



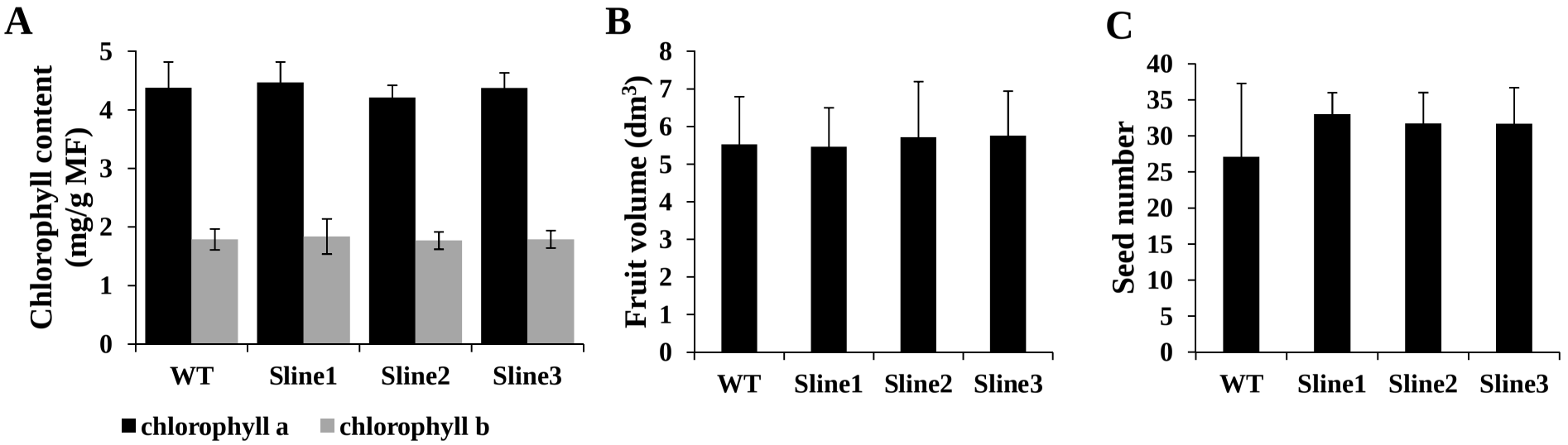
Supplementary Figure S2. Transcript levels of *Sl-IAA27* gene in three independent *Sl-IAA27* over-expressing lines assessed in leaves by qRT-PCR.
over-expressing lines = Sline1, Sline2, Sline3

Fig S3



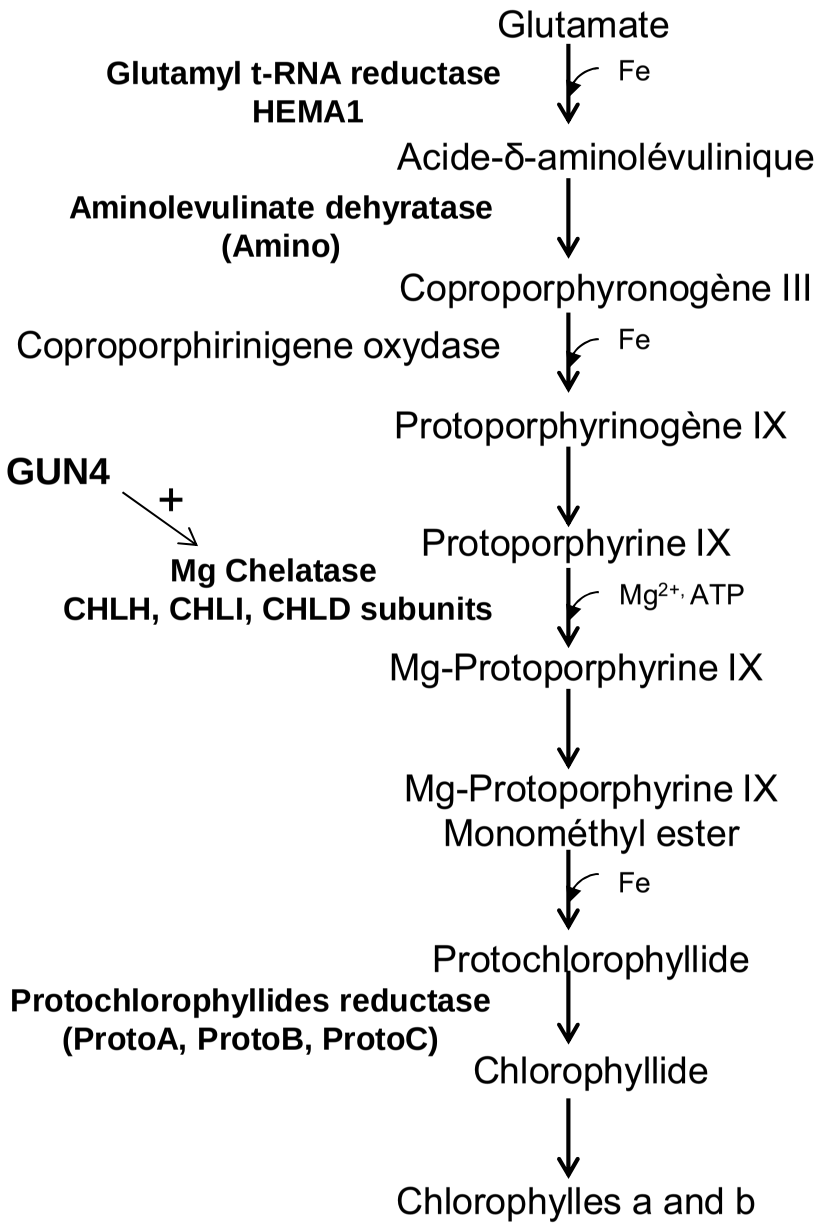
Supplementary Figure S3. Altered root growth in *Sl-IAA27* over-expressing lines. (A) Root development in WT and *Sl-IAA27* over-expressing lines assessed in three week-old seedlings grown on MS/2 medium. (B) Mean of primary root length in WT and *Sl-IAA27* over-expressing lines. (C) Effect of NPA (5µM) treatment on root development of three week-old wild type (WT) and *Sl-IAA27* over-expressing lines grown on MS/2 medium.
over-expressed lines =Sline1, Sline2, Sline3

Fig S4



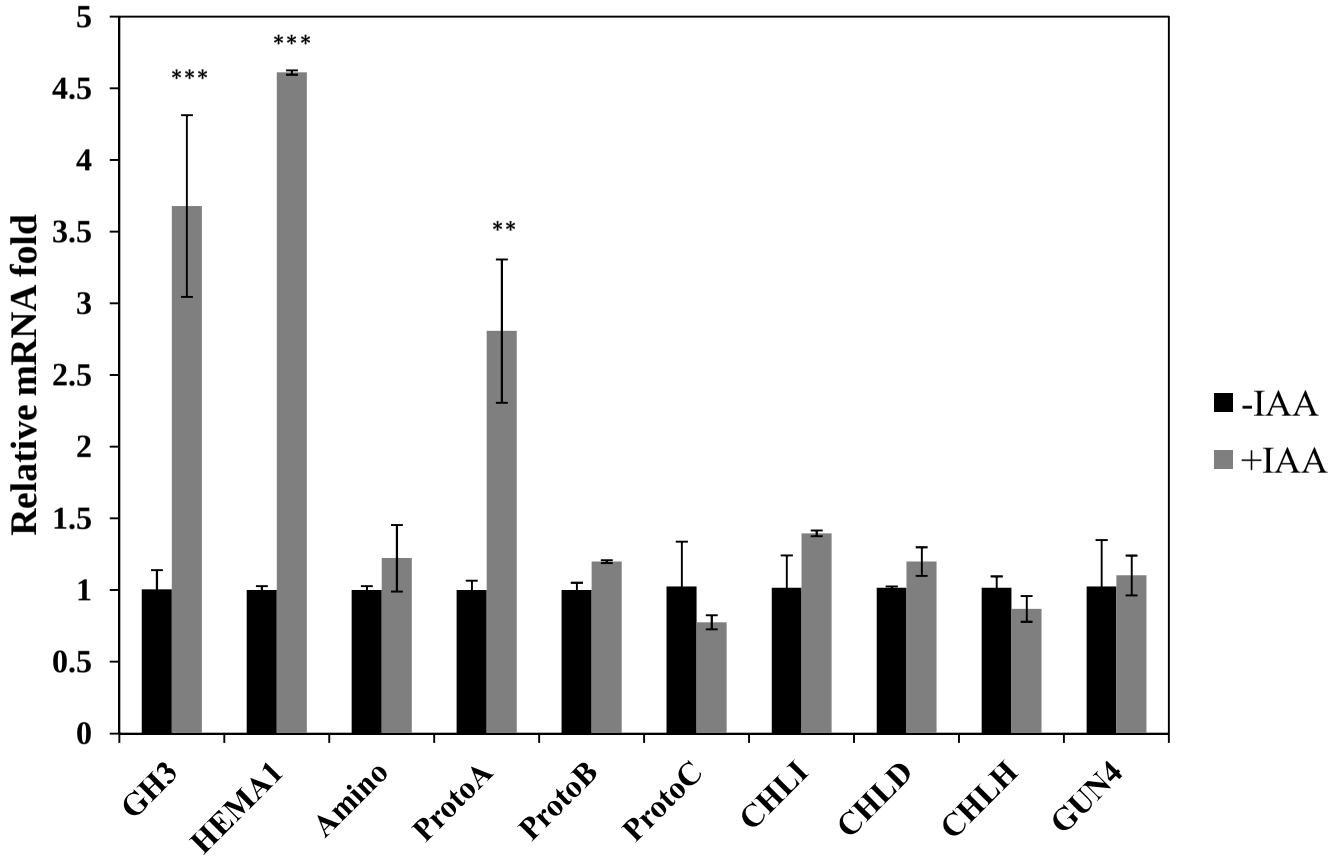
Supplementary Figure S4. *Sl-IAA27* over-expressing lines. (A) Chlorophyll a and b leaf content (mg/g Fresh weight (FW)) in *Sl-IAA27* over-expressed and WT lines assessed by spectrophotometry. (B) Fruit volume determined at full-ripe stage (BK+10) of WT and *Sl-IAA27* over-expressed lines. (C) Seed number in WT and *Sl-IAA27* over-expressed mature fruits. Over-expressing lines= Sline1, Sline2, Sline3

Fig S5



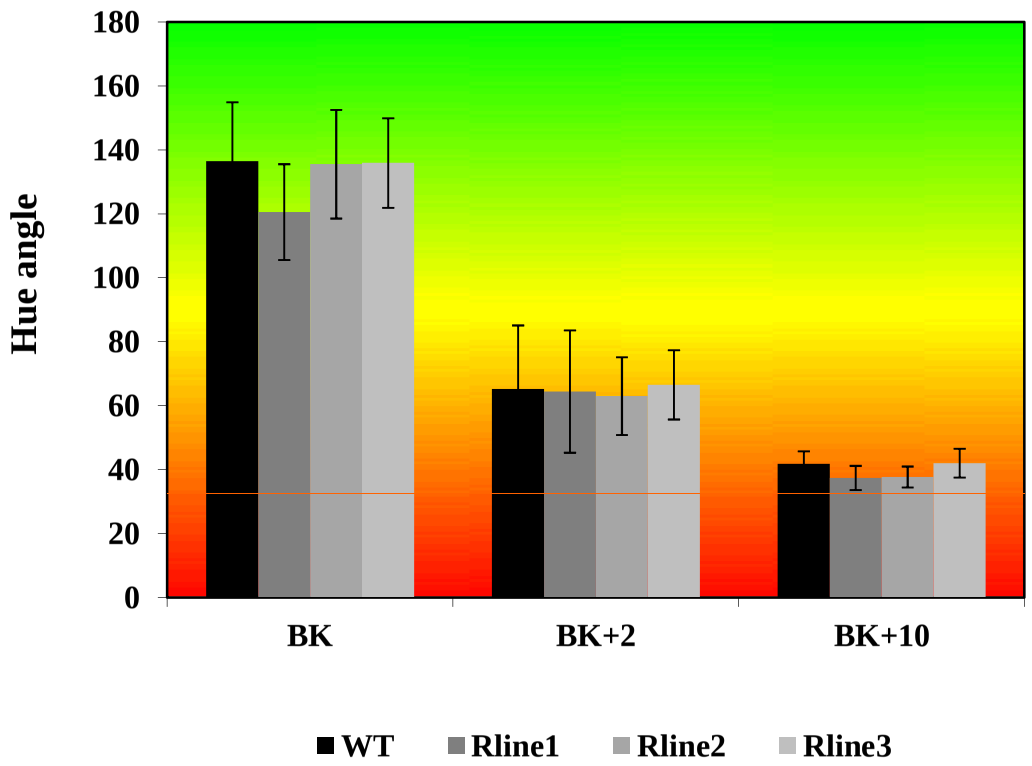
Supplementary Figure S5. Chlorophyll a and b biosynthesis pathway

Fig S6



Supplementary Figure S6. Auxin regulation of chlorophyll biosynthesis pathway. Transcript accumulation monitored by qRT-PCR in wild type seedlings. mRNA levels upon auxin treatment are represented by grey boxes compared to normal conditions, used as reference, in black. Auxin treatement was performed during 2h with NAA at 20µM. Statistical analysis were realized using student test, ***p-value<0.001, ** p-value <0.01, * p-value < 0.05.

Fig S7



Supplementary Figure S7. Evolution of fruit color during ripening at breaker (BK), breaker and two days (BK+2), breaker and ten days (BK+10) for wild type (WT) and Sl-IAA27 RNAi lines. Hue angle was determined using Chroma Meter CR-310 Minolta. 60 fruits per line were used for experiment.

Supplementary tables

Table S1. SGN number, EST number, chromosome localization, ITAG release 2.3 number and AuxRE elements in promoter of the tomato genes analyzed related to chlorophyll biosynthesis

Gene name	SGN-Unigene	EST number	K	ITAG release 2.3	TGTCTC	TGTCCC
glutamyl t-RNA reductase HEMA1	SGN-U579959	59	chr04	Solyc04g076870.2.1	3	0
Aminolevulinate dehydratase	SGN-U579023	50	chr08	Solyc08g069030.2.1	0	0
Protochlorophyllide reductase A	SGN-U578669	80	chr07	Solyc07g054210.2.1	1	2
Protochlorophyllide reductase B	SGN-U577510	107	chr12	Solyc12g013710.1.1	1	2
Protochlorophyllide reductase C	SGN-U580321	88	chr10	Solyc10g006900.2.1	0	1
Magnesium chelatase CHLI subunit	SGN-U573205	111	chr10	Solyc10g008740.2.1	0	0
Magnesium chelatase CHLH subunit	SGN-U581974	59	chr03	Solyc03g118240.2.1	1	1
Magnesium chelatase CHLHD subunit	SGN-U570841	19	chr04	Solyc04g015490.2.1	0	1
GUN4	SGN-U568307	11	chr06	Solyc06g073290.1.1	0	0

Table S2. Quantitative PCR primers of all genes analyzed in this article.

Gene Name	Primer Sequence	SGN number
Actin	F 5'-TGTCCCTATCTACGAGGGTTATGC-3' R 5'-AGTTAAATCACGACCAGCAAGAT-3'	SGN-U580609
Sl-IAA1	F 5'-TGAATCTAAGTCAAGTTCTGATCATGTC-3' R 5'-ATGATGTTTTTCCTGTTAGATCTCACTG-3'	SGN-U579410
Sl-IAA2	F 5'-TAACAATGATGAACCACCAC-3' R 5'-TTTCCTTAAATAAGCCGCAC-3'	SGN-U599474
Sl-IAA3	F 5'-ATATAATGGATCTGATTATGCACCAACA-3' R 5'-TTATAAACATCTCCCATGGTACATCAC-3'	SGN-U577993
Sl-IAA4	F 5'-AACAAGAGGGCTTTGCCTGAG-3' R 5'-GTGTCTTGGCAACAGGTGGA-3'	SGN-U579749
Sl-IAA7	F 5'-ACTCAACCTCCATCATAATGATAATATTCC-3' R 5'-ACCCCACCACTTGAGCCTTA-3'	SGN-U579168
Sl-IAA8	F 5'-ATTCTGCTACTTTGATAATCTTGCACA-3' R 5'-TGTCCATTGATGAAACACAGCTCT-3'	SGN-U581702
Sl-IAA9	F 5'-CCCCTTGCACCCTTCCA-3' R 5'-AGCGTCTGAAAATCCTCGTTTG-3'	SGN-U568849
Sl-IAA11	F 5'-GGAGATGTTCTTCAAATCAACC-3' R 5'-TCTGATGATCCATCCAAGAG-3'	SGN-U577813
Sl-IAA12	F 5'-CCACGCGATCTTCAGCATAA-3' R 5'-TCTGTTTCAGGGAGCGGC-3'	SGN-U579795
Sl-IAA13	F 5'-AGTCTTTTAAGCTCTTGGATGGATCA-3' R 5'-AAACATCCCGAATGGAACATCT-3'	SGN-U579354
Sl-IAA14	F 5'-GTTTACGCATAATGAAAGGATCAGAAG-3' R 5'-TTATCTATGGAGCTTGCACACCA-3'	SGN-U579618
Sl-IAA15	F 5'-CCTAACAATCTGTAATTCTCAAAGTGAAA-3' R 5'-GCATCCAGTCTCCATCTTTATCTTC-3'	SGN-U579568
Sl-IAA16	F 5'-GCGTGTTGGGTGCGGA-3' R 5'-CGATTCCAGTTCATTCCCATTAG-3'	SGN-U580151
Sl-IAA17	F 5'-CAAGAATTATTTGATGCCTTAACCAA-3' R 5'-ACTATTCAAAAGGTCCATCAGTTTCC-3'	SGN-U593495 SGN-U581524
Sl-IAA19	F 5'-TGTCGGCGATGTTCCATG-3' R 5'-AAGTCTCTTGCTCCAAGCCCTAT-3'	SGN-U579607
Sl-IAA26	F 5'-AAAGGCTGCGTGTGTTGAAA-3' R 5'-CAAGATCTGTTGGCTCTACATCTTGT-3'	SGN-U573372
Sl-IAA27	F 5'-CCAAAAAGAGGGAATGGAGGTT-3' R 5'-TGTTCTCCCTTCATCATCATTTTTC-3'	SGN-U577682 SGN-U580267
Sl-IAA29	F 5'-GGTTTTGATGATAGCTTCTCCGATA-3' R 5'-ACGTCTTACGTTCAACTACTCCTTCA-3'	SGN-U568970
Sl-IAA36	F 5'-AGAAATTCCATGTATGTGAAGGTTAAAA-3' R 5'-CATTTGGAGCAAAGTGTTAGTAAGGA-3'	SGN-U586760
GH3	F-5'AGCTCGTCATCACAACATACGC-3' R-5'GTTTATCGACAAGGGCGAGTTG-3'	SGN-U573533
Glutamyl t-RNA reductase HEMA1	F 5'-CAGTATCCACACTACGCCTG-3' R-5'CAGGATGGAGATCTATGTTGTG-3'	SGN-U579959
Aminolevulinate dehydratase	F-5'GTAATCGTAGGTCGTCAGCA-3' R-5'TGGATGTTATAGGCTTGGATGGA-3'	SGN-U579023
Protochlorophyllide reductase A	F-5'GCAATCACCCAGTCTACCTC-3' R-5'GGCTATAGGCGAGTCAGGAG-3'	SGN-U578669
Protochlorophyllide	F-5'ACTAACCATCTTGGTCATTTCC-3'	SGN-U577510

reductase B	R-5'GGAAACACAAACACATTGGCT-3'	
Protochlorophyllide reductase C	F-5'CAACAACAGGGCTATTCAGAG-3' R-5'CTGAATCTGGAAAGAGACTTGC-3'	SGN-U580321
Magnesium chetalase CHLI subunit	F-5'GAATATCAATTTCACACCCTGCTC-3' R-5'CAGAACTAAGAGTGAAGATCGTC-3'	SGN-U573205
Magnesium chetalase CHLH subunit	F-5'AAGAAGGTGCCATTGTATCAG-3' R-5'GACTTGGAGAGTTTGGATGG-3'	SGN-U581974
Magnesium chetalase CHLD subunit	F-5'CCATTGACCGTGAGATAGGA-3' R-5'CTGAAGAGTGGGAAGATGGG-3'	SGN-U570841
GUN4	F-5'AGTTCATTTCCGAATCTGACC-3' R-5'GGAATAACAAAGCGAACAGAGA-3'	

III Complementary results

III.1 Ethylene regulation of *Sl-IAA27*

III.1.1 Material and Methods

Ethylene treatment

Seeds were sown on plates with MS/2 medium and grown in dark during one week. For each transgenic line and wild type four plates of 40 seeds were analyzed, two plates were treated 24 h with 10 ppm of ethylene and two plates were let in normal conditions. For real-time PCR analysis seedlings were then frozen. RNA extractions, cDNA synthesis and real-time experiment were harvested as described previously.

Anaylsis of ethylene production in fruit

Ethylene production of fruit was analyzed thanks to chromameter after have been let the fruit 30 min in closed recipient.

III.1.2 Results

The results presented in the chapter II include ethylene regulation of the expression of ***Aux/IAA*** genes analyzed in seedlings of ***Ailsa Craig*** cultivar. The physiological significance of the ***Sl-IAA27*** gene was mainly investigated through the analysis of transgenic plants generated from the wild type cultivar ***MicroTom***. Therefore we determined if the ***Sl-IAA27*** regulation of expression upon ethylene treatment is the same in the ***MicroTom*** cultivar. The analysis of ***Sl-IAA27*** expression in ***MicroTom*** seedlings treated with ethylene showed that like in ***Ailsa Craig*** the ethylene down-regulates ***Sl-IAA27*** expression (Figure 32).

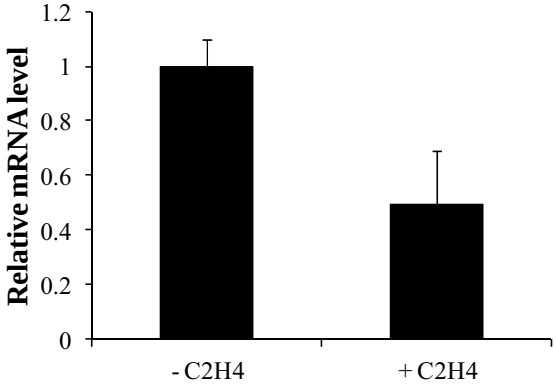


Figure 30: Ethylene regulation of *Sl-IAA27* expression. *Sl-IAA27* transcript accumulation was monitored by qRT-PCR in dark-grown one week old seedlings treated 24 h with 10 ppm of C_2H_4.

Ethylene is known to enhance apical hook curvature in dark-grown seedlings leading cotyledons to form a 270° bend relative to the hypocotyls (Ecker 1995). This phenotype is one of the hallmarks of the classical ethylene triple response together with reduced hypocotyls and root elongation (Ecker 1995; Bleecker and Kende 2000). To determine if the modification of ***Sl-IAA27*** expression leads to an altered triple response seedlings of both ***Sl-IAA27*** over-expressing and RNAi plants were treated with ethylene. Results showed that all plants display triple response with notably similar hook curvature compared to wild type plants (Figure 33).

Figure 31: Triple response phenotype of dark-grown one week seedlings of *Sl-IAA27* over-expressing plants (Sline1 and Sline2) and *Sl-IAA27* RNAi (Rline1, Rline2, Rline3). Seedlings were treated 24 h with 10 ppm of ethylene.

The ethylene is known as a key regulator of climacteric fruit ripening such as tomato fruit. To determine if the modification of ***Sl-IAA27*** expression leads to an alteration of ethylene production during fruit ripening we determined fruit production of ethylene at three stages of ripening in both ***Sl-IAA27*** over-expressing and RNAi plants. No significant difference was observed either in ***Sl-IAA27*** over-expressing fruits or in ***Sl-IAA27*** RNAi fruits compare to wild type fruits at the same stage of ripening (Figure 34).

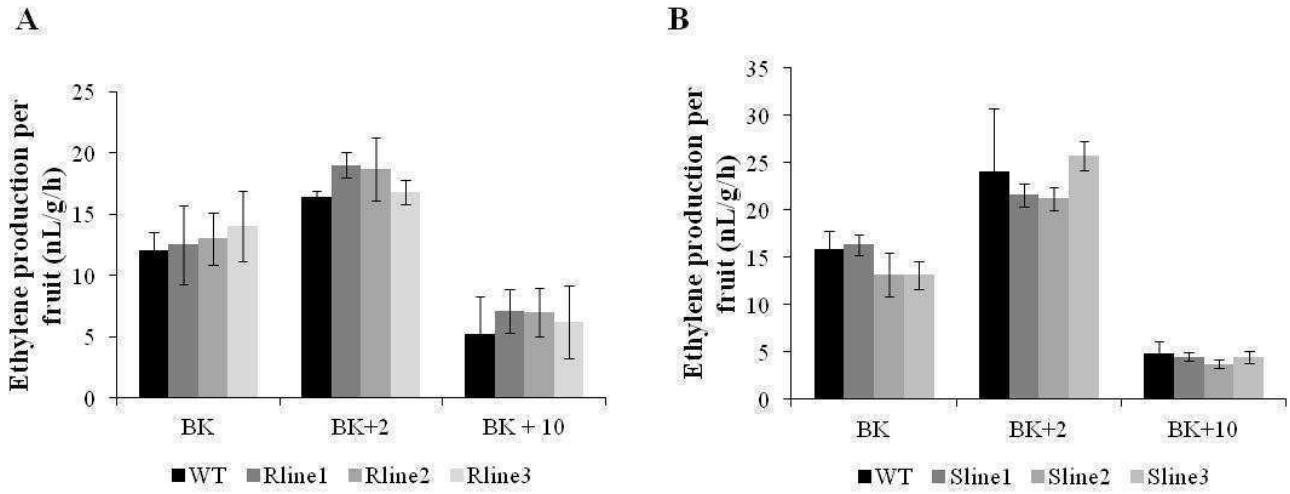


Figure 32: Ethylene production of *Sl-IAA27* transgenic fruits during ripening at breaker (BK), breaker and two days (BK+2) and breaker and ten days (BK+10). (A) Ethylene production in *Sl-IAA27* RNAi lines (Rline1, Rline2, Rline3) and in wild type fruits. (B) Ethylene production in *Sl-IAA27* over-expressing lines (Sline1, Sline2, Sline3) an in wild type fruits.

In conclusion, no ethylene related phenotype in ***Sl-IAA27*** transgenic plants.

III.2 Protein interaction between Sl-IAA27 and Sl-ARFs

III.2.1 Material and Methods

Yeast-two hybrid experiment:

Sl-IAA27 CDS (coding sequence) and ***Sl-ARFs*** CDS were cloned by Gateway technology in plasmids deriving from pGAD and pGBK vectors (Clontech, Washington, United States). For control Y2H experiments, yeasts were co-transformed with the pAD SV40T (Clontech) and the pBD-p53 (Clontech). Transformations were performed thanks to LiAC and PEG 50% using Yeast AH109 competent cells and 1µg of each plasmid. For each transformation, yeasts were selected on minimal SD base medium (Clontech) supplemented in amino acids with exception of tryptophane, histidine (SD-TH) and additionally leucine (TLH) or adenine (TLHA).

Pull-down assay: from protein production to western blot

The ***Sl-IAA27*** CDS, ***Sl-ARF8*** CDS and ***Sl-ARF4*** CDS were cloned by Gateway in pDest15 and pDest17 vector in frame with GST and HIS tags and introduced into the Rosetta strain. For control, a GST protein was produced after transformation with a pGEX-3X vector (Amersham, Pantin, France). Bacteria were cultivated in LB until OD_{600} reached 0.6. Protein expression was then induced upon addition of IPTG (0.1 mM final concentration). After 4 hours of culture at 20° (Sl-ARF8) or 28°C (Sl-IAA27**,** GST and Sl-ARF4), bacteria were collected by centrifugation (10000g, 10min), frozen in liquid nitrogen and stored at -80°C. Frozen bacteria were resuspended in Phosphate Saline Buffer (PBS: 140 mM NACl, 2.7 mM KCl, 10 mM NA_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3), ground in a French Press (Thermo) and sonicated (2x30s). After centrifugation (30min 10000g) GST-tagged bait proteins (Sl-IAA27 and GST) were purified on a Gluthatione Sepharose Fast Flow (Amersham) according to manufacturer instructions and desalted in PBS buffer on NAP-5 columns (Amersham). Prey proteins were obtained from the crude soluble extract obtained after centrifugation. For pull-down assay, the MagneGST Protein Purification system (Promega) was used. For each assay,

15 µg of purified bait proteins diluted in MagneGST binding buffer (final volume equals to 100 µL) were immobilized on MagneGST Glutathione Particles. After rinsing with MagneGST wash buffer, particles were incubated 1h at RT with 200 µL of prey proteins crude soluble extracts. After rinsing with MagneGST Binding wash buffer, proteins were eluted using 1x SDS and supernatant were used for western blot analysis. Western blot (1h at 100V) was realized after sample migration on 12% acrylamide gel (1h30min at 150V). Antibodies anti-HIS-Hrp (Roche) were used for detection.

III.2.2 Results

The auxin signal is in part modulated by the quantitative and qualitative responses of the Aux/IAA repressors and the ARF transcription factors. Multiple lines of evidence clearly indicate that different expression patterns and different affinities among these two protein families both contribute to the Aux/IAA–ARF system (Hayashi K, 2012). In Arabidopsis, a yeast two-hybrid experiment indicated that there are specific pairs of interactions between the Aux/IAA and ARF proteins (Vernoux et al. 2011). To determine with which Sl-ARFs the Sl-IAA27 protein is able to interact, yeast-two hybrid (Y2H) experiments were performed. Interaction was tested with 14 Sl-ARFs including 4 Sl-ARFs activators and 10 Sl-ARFs repressors (M. Zouine, personnal communication). The results showed that Sl-IAA27 is able to interact with all Sl-ARF activators tested (ARF5, ARF6a, ARF7, ARF8a and ARF8b) but not with Sl-ARF repressors (Table 1).

Table 1: Screening by Yeast-two hybrid experiment of Sl-IAA27 interaction with Sl-ARFs. ARF activators are indicated in green and ARF repressors in blue.

	ARF1	ARF2a	ARF2b	ARF3	ARF4	ARF5	ARF6a	ARF6b	ARF7	ARF8a	ARF8b	ARF9a	ARF9b	ARF10	ARF17
IAA27	no	no	no	no	no	yes	yes	no	yes	yes	yes	no	no	no	no

To confirm Y2H data Sl-IAA27 interaction with Sl-ARF8 (activator) and Sl-ARF4 (repressor) was investigated ***in vitro*** using pull-down assay. Similarly than in yeast, pull-down showed that Sl-IAA27 is able to interact with Sl-ARF8 but not with Sl-ARF4 (Figure 35).

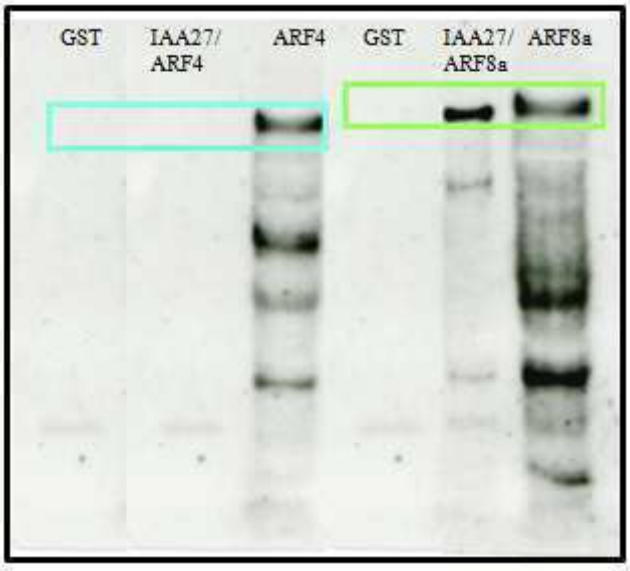


Figure 33 : Western blot analysis of Sl-IAA27 interaction with Sl-ARF4 and Sl-ARF8 tested by pull-down assay. The GST alone was used as negative control. Sl-ARF4 and Sl-ARF8 proteins were used as positive control of western blot.

III.3 Protein interaction between Sl-IAA27 and TOPLESS

III.3.1 Material and Methods

Identification of TOPLESS tomato homologs:

TOPLESS homologs in tomato were identified by BLASTN analysis performed on the tomato genome using TOPLESS from Arabidopsis.

Yeas-two hybrid experiment:

As for Sl-ARFs the six ***TOPLESS (TPL 1-6)*** CDS identified in the tomato genome and Sl-IAA29 CDS were cloned by Gateway in plasmids deriving from pGAD and pGBK vectors (Clontech, Washington, United States). Protocol experiment was similar to that used for Sl-ARFs interaction screening.

BIFC experiment:

The Sl-IAA27 CDS, Sl-ARF8 CDS and Sl-TPL5 CDS sequence were cloned in frame with YFP amino (N) and carboxyl (C) terminal parts by Gateway technology in pAM-YFPN and pAMP-YFPC vectors. Protoplasts were obtained from suspension-cultured tobacco (***Nicotiana tabacum***) BY-2-cells and transfected according to the method described previously (Leclercq et al. 2005). YFP fluorescence measurement by flux cytometry was performed as described previously (Audran-Delalande et al. 2012).

III.3.2 Results

The TOPLESS (TPL) proteins were shown in Arabidopsis to be able to interact with Aux/IAA proteins notably through conserved domain I, playing the function of co-repressors of auxin signaling. In tomato six TPL homologs were identified (conjugated work with Yanwei Hao and Mohammed Zouine) (Table 2). In addition Sl-IAA27 protein displays a conserved domain I and efficient repressor activity. To determine if Sl-IAA27 can interact with the TPL proteins (TPL 1-6), Y2H experiments were performed. Results showed that among the six TPL, Sl-IAA27 can directly interact with TPL1, 3 and 5 (Figure 36).

Table 2: SGN number, EST number, chromosome localization and ITAG release 2.3 number of TOPLESS homologs identified in tomato

Gene name	SGN unigene	EST number	K	ITAG release 2.3
Topless1	SGN-U580074/SGN-U574388	4/13	chr03	Solyc03g116750.2.1
Topless2	no	no	chr08	Solyc08g029050.2.1
Topless3	SGN-U580205/SGN-U574706/SGN-U580159	25/5/4	chr03	Solyc03g117360.2.1
Topless4	SGN-U600982/SGN-U585395/SGN-U575076	1/2/4	chr07	Solyc07g008040.2.1
Topless5	SGN-U585392	10	chr08	Solyc08g076030.2.1
Topless6	SGN-U566564/SGN-U570520	20/3	chr01	Solyc01g100050.2.1

Studies realized in Arabidopsis showed that Topless-Aux/IAA interaction is specific to the Aux/IAA analyzed. To see if Sl-IAA proteins display similar behavior the interaction with TPL proteins was also tested with Sl-IAA29. Results showed no interaction between TPL and Sl-IAA29 (Figure 36), suggesting that like in Arabidopsis the Aux/IAA-TPL interactions are specific.

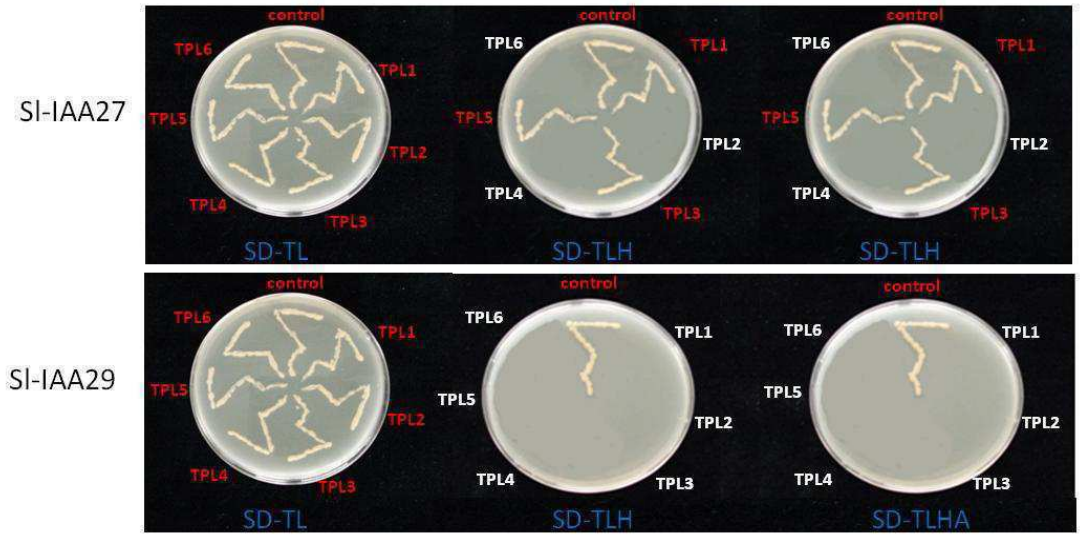


Figure 34 : Screening by Yeast-two hybrid of Sl-IAA27, Sl-IAA29 and Sl-IAA26 interaction with TOPLESS. TOPLESS=TPL. When interaction was observed the protein name was written in red.

Moreover, to confirm data obtained by Y2H experiment regarding Sl-IAA27 interaction with TPL, BIFC experiments were performed. As Sl-IAA27 was shown to interact with Sl-ARF8,

this protein was used as positive control. Analyses of YFP fluorescence revaled like by Y2H experiment an interaction between Sl-IAA27 and TPL5 but not between Sl-IAA27 and TPL2 (Figure 37).

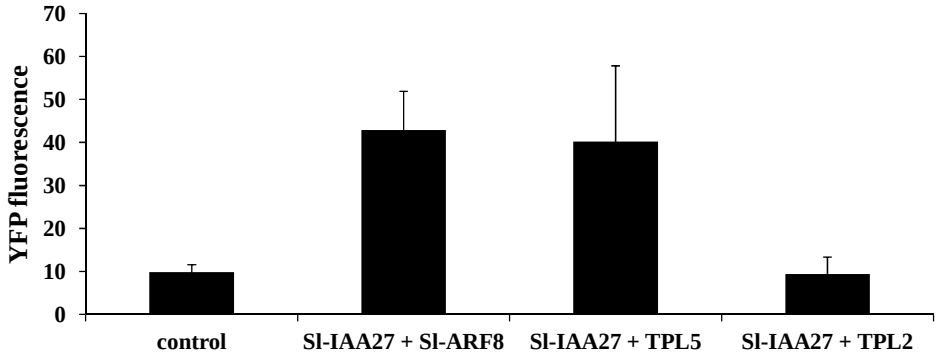


Figure 35: Analysis of Sl-IAA27 and TPL5 interaction by BIFC method. Empty vector was used as negative control and Sl-ARF8 as positive control.

III.4 Conclusion

Phenotypic analysis of ***Sl-IAA27*** down-regulated plants revealed that normal expression of ***Sl-IAA27*** is required for chlorophyll biosynthesis and normal flower and fruit development. While the expression of ***Sl-IAA27*** is down-regulated by ethylene no related phenotype has been observed in transgenic plants. This suggests that either ***Sl-IAA27*** is not directly involved in ethylene regulation or that its function in ethylene pathway can be replaced by another ***Sl-IAA*** or molecular component. The silencing of ***Sl-IAA27*** leads to the down-regulation of many genes related to chlorophyll biosynthesis suggesting that in normal conditions Sl-IAA27 represses ARF repressors activity regulating chlorophyll biosynthesis pathway. Nevertheless, yeast-two hybrid experiments performed revealed that Sl-IAA27 is only able to interact directly with Sl-ARFs activators. We can therefore hypothesize that Sl-IAA27 represses ARFs repressors by an indirect interaction. Analysis of Sl-IAA27 interaction with TPL

homologues in tomato showed direct interaction between Sl-IAA27 and TPL 1, 3 and 5. In Arabidopsis the TPL proteins were shown to be able to interact with ARFs either repressors or activators and notably to mediate At-IAA12 interaction with At-ARF5 and At-ARF7. In consequence TPL 1, 3 and 5 could be necessary to mediate interaction between Sl-IAA27 and ARFs repressors notably in leaves.

General conclusions and perspectives

Auxin is a central hormone that exerts pleiotropic effects on plant growth including the development of roots, shoots, flowers and fruit. The perception and signaling of the plant hormone auxin rely on the cooperative action of several components among which Aux/IAAs play a pivotal role. During this work, we identified and comprehensively analysed the entire *Aux/IAA* gene family in tomato (*Solanum lycopersicum),* a reference species for Solanaceae plants, and the model plant for fleshy fruit development. The main part of my PhD was focused on the functional characterization of one member of this family, the *Sl-IAA27* gene.

The *Aux/IAA* multigene family in tomato

We have identified 25 tomato *Aux/IAA* genes and they were renamed to comply with the nomenclature of their closest Arabidopsis homologs. The tomato *Aux/IAA* genes family gathers a lower number of genes than in Arabidopsis (29 genes) (Liscum and Reed 2002). Notably, the tomato genome contains 2 non-canonical *Aux/IAA* genes (*Sl-IAA32* and *Sl-IAA33*) whereas up to 6 are found in Arabidopsis. Despite that the over-expression of *AtIAA20*, *AtIAA30* or *AtIAA31* results in aberrant auxin-related phenotypes in Arabidopsis (Sato and Yamamoto 2008), the mechanism by which the non-canonical proteins impact auxin signaling remains unclear. All previous studies realized showed that Aux/IAAs are addressed to nucleus. We have demonstrated for the first time that an Aux/IAA (Sl-IAA32) could also be addressed to the cytoplasm. The Sl-IAA32 protein lacks conserved domain II required for the degradation of the protein mediated by the Auxin/TIR1 complex and a bipartite NLS. Nevertheless it displays conserved domain I and was shown to be a functional repressor of auxin signaling. Its expression is limited to the breaker stage of fruit development. Overall its probably atypical long-lived feature due to the absence of domain II and its extra-nuclear localisation suggest that Sl-IAA32 and more generally non-canonical

Aux/IAA proteins may have specific function in mediating auxin responses during specific plant developmental events. This remains to be elucidated.

In previous reports, it has been demonstrated that Aux/IAAs display a repression activity of auxin-dependent transcription (Ulmasov et al. 1997, Tiwari et al. 2001, Bargmann and Birnbaum 2009). However, our study is the first analyse dealing with the repression activity of a whole Aux/IAA proteins family. In tomato, the repression levels vary widely (23% to 87%) among Aux/IAA proteins when tested with the synthetic DR5 promoter. It has been previously described in Arabidopsis, that domain I of Aux/IAA proteins is an active repression domain containing the LxLxL motif (Tiwari et al. 2004) that interacts with the TOPLESS (TPL) co-repressor (Szemenyei et al. 2008). All the tomato Aux/IAAs tested in this study bear a conserved domain I, but no correlation was found between the level of repression and the amino acid environment surrounding the LxLxL repressor motif, neither the length of the repression domains (e.g. an LxLxL versus an LxLxLxLxL motif) nor the presence of two LxLxL motifs in the same Aux/IAA protein. Recently, it has been reported that mutations in domain I of various Aux/IAA proteins can have profound, but different consequences in terms of auxin responses in Arabidopsis plants suggesting that some Aux/IAA proteins may have stronger or more complex repression domains than others (Li et al. 2011). Dedicated tomato mutant resources are now needed to better understand the intrinsic differences in the repression domains of Sl-IAA proteins and to better clarify the functional significance of the diversification of Aux/IAA members between tomato and Arabidopsis.

The expression patterns of *Sl-IAA* genes in various tissues and organs suggest that the encoded proteins may perform both specific and redundant functions. No link was found

between the clustering based on the expression pattern and the clustering obtained by phylogenetic analysis. Accordingly, the six members of the Pt*IAA3* subgroup in *Populus trichocarpa* are differentially expressed (Kalluri et al. 2007) and in Arabidopsis, gene expression patterns of *Aux/IAA* sister pairs are significantly different (Paponov et al. 2009). These data support the idea that the diversification of *Aux/IAA* family members in flowering plants has been also sustained by changes in their expression patterns. In Arabidopsis, using a promoter-swapping strategy, it has been shown that the physiological function of Aux/IAA proteins was determined by both the pattern of gene expression and the properties of proteins (Weijers et al., 2005; Muto at al., 2007). Similar studies should be performed in the tomato.

The study of *Sl-IAA* patterns of expression upon auxin or ethylene treatment revealed that most of *Sl-IAAs* genes could be regulated by both hormones. This suggests that *Sl-IAAs* could be a molecular link between the ethylene and auxin signaling pathways. This is in accordance with previous report on *Sl-IAA3* characterization which showed that down-regulation of this gene leads to both auxin and ethylene related phenotypes (Chaabouni et al. 2009a and Chaabouni et al. 2009b). Nevertheless, the potential role of the ethylene-regulated *Aux/IAA* genes in mediating the cross-talk between auxin and ethylene remains to be further investigated in particular during developmental events such as the transition from green-to-ripe fruit where ethylene is known to be a key player.

Functional characterization of the *Sl-IAA27* gene

The *Sl-IAA27* gene displaying both auxin and ethylene regulated expression it appeared to be a suitable candidate gene to analyze the cross-talk between auxin and ethylene during fruit development. Indeed, the expression of *Sl-IAA27* is down-regulated by both hormones. Therefore *Sl-IAA27* displays a distinct behavior compared to most *Aux/IAAs* regarding the

regulation of its expression by auxin. Moreover, *Sl-IAA27* expression is down-regulated during fruit ripening while ethylene production increases. Interestingly, the Sl-IAA27 protein is the closest homologue to Sl-IAA9 which was shown to be a key regulator of tomato fruit set (Wang et al. 2005). Information regarding Sl-IAA27 function was lacking either in the Solanaceae or in Arabidopsis. Tomato transgenic plants under-expressing the *Sl-IAA27* gene revealed multiple phenotypes related to vegetative and reproductive growth. Silencing of *Sl-IAA27* results in higher auxin sensitivity, altered root development and reduced chlorophyll content in leaves. In line with the reduced chlorophyll content in *Sl-IAA27* RNAi leaves, genes involved in chlorophyll synthesis display lower expression at the level of transcript accumulation. Both ovule and pollen display dramatic loss of fertility in *Sl-IAA27* down-regulated lines and the internal anatomy of both flower and fruit are modified with enlarged placenta in smaller fruits.

The smaller size of the *Sl-IAA27* down-regulated fruits may result from the reduced photosynthetic activity in the leaves. This hypothesis is supported by the prevailing opinion that fruit growth is predominantly allowed by photoassimilate supply from source tissues (Lytovchenko et al. 2011). Our results showed that Sl-IAA27 could control the photosynthesis process by regulating chlorophyll biosynthesis. To test if primary metabolism is modified by the silencing of *Sl-IAA27* gene, metabolomic analyses will be performed on leaves and developing fruits of *Sl-IAA27* RNAi plants (collaboration with Yves Gibon, CGFB metabolome platform, Bordeaux, France).

While the expression of *Sl-IAA27* is down-regulated by ethylene no ethylene related phenotype was observed either in *Sl-IAA27* over-expressing plants or *Sl-IAA27* RNAi plants.

This suggests that *Sl-IAA27* may be not directly related to ethylene pathway or that its function is redundant with other molecular components.

At the molecular level, the identification of Sl-IAA27 protein partners is a prerequisite to understand the signaling pathway depending of this protein. Yeast-two hybrid experiments indicated that Sl-IAA27 protein can interact directly with Sl-ARFs activators and with three Sl-TPLs (TPL1, 3 and 5). These data were in part verified by *in vitro* analysis with pull-down assay and by *in vivo* analysis in tobacco protoplasts by BIFC respectively. In Arabidopsis the TPL proteins were shown to be able to interact with ARFs either repressors or activators and notably to mediate At-IAA12 interaction with At-ARF5 and At-ARF7. Overall it suggests that Sl-IAA27 could also interact with Sl-ARFs repressors through complex formation with TPL proteins (TPL 1, 3 or 5). This would be in line with the hypothesis that Sl-IAA27 regulates chlorophyll biosynthesis by repressing the activity of ARFs repressors which control this process. Y2H experiments are currently performing to determine if Sl-ARFs repressors are able to directly interact with TPL proteins. In this case thus interacting with TPL 1, 3 and 5 should be suitable candidate to perform pull-down assay experiment with TPLs and Sl-IAA27 protein together. Interestingly, Sl-IAA27 is able to interact with Sl-ARF8 which is implicated in tomato fruit development (M. Zouine, personal communication). To determine if Sl-IAA27 function in regulating fruit development is mediated at least in part through the repression of Sl-ARF8 activity, it would necessary to determine if these proteins are interacting in tomato fruits. Until now only direct interactions with selected candidate were investigated. To have a general overview on the Sl-IAA27 interactome, non-targeted approaches will be needed like the use of BIACORE technology or co-immunoprecipitation experiments. Indeed, the generation of transgenic plants with tagged Sl-IAA27 protein could allow the identification of proteins or proteins complexes in a specific tomato tissue.

Overlapping and specific functions of *Sl-IAA* in tomato

To date, the functional characterization of four *Sl-IAAs* has been performed in our laboratory and in collaboration with the laboratory of Z. Li (Chongqing University, China). The results show that *Sl-IAAs* display both overlapping and specific functions as it was described in Arabidopsis. Nevertheless, in tomato the elucidation of *Aux/IAA* function comes from the characterization of loss of function mutants whereas analyses of this type of transgenic lines were almost inefficient in Arabidopsis, probably due to a large redundant function of the *Aux/IAAs* in this specie.

Previous report on *Sl-IAA9*, *Sl-IAA15* and the study of *Sl-IAA27* presented here showed that *Sl-IAAs* are involved in the regulation of flower and fruit development but at different step. *Sl-IAA9* is able to trigger fruit initiation prior to fertilization (Wang et al. 2005) while *Sl-IAA27* seems required to maintain proper ovule development and fertilization capacity. The *Sl-IAA15* gene appears also as a regulator of fruit development its down-regulation resulting in decreased fruit set (Deng et al. 2012). Interestingly, characterization of *Sl-IAA3,* which expression is clearly regulated during fruit ripening, did not reveal any physiological significance of this gene in fruit development (Chaabouni et al. 2009a). In addition, *Sl-IAA* genes mediate tomato vegetative development through different way. At the exception of *Sl-IAA27* RNAi lines, down-regulation of *Sl-IAA* leaded in each case to altered dominance apical with modified axillaries shoots formation and stem elongation (Wang et al. 2005; Chaabouni et al. 2009a; Deng et al. 2012,). In leaves, the expression of *Sl-IAA9* is necessary to maintain the formation of compound leaves (Wang et al. 2005). The down-regulation of *Sl-IAA27* leads to reduced chlorophyll content but under-expression of *Sl-IAA15* results in dark green and thick leaves formation (Deng et al. 2012). In addition, down-regulation of *Sl-IAA15* results in

lower trichome number but such a phenotype was never observed in other down-regulating *Sl-IAA* plants (Deng et al. 2012). In roots, silencing of *Sl-IAA9*, *Sl-IAA15* and *Sl-IAA27* result in increased lateral root formation.

During my PhD, phenotypic analyses were also performed on *Sl-IAA16*, *Sl-IAA17* and *Sl-IAA29* RNAi lines. No phenotype was observed on *Sl-IAA16* RNAi plants but fruits of *Sl-IAA17* RNAi plants displayed abnormal development. The functional characterization of the *Sl-IAA17* gene is actually performed by Lyan Su during her PhD. First study of *Sl-IAA29* RNAi showed an alteration of flower and fruit morphogenesis with the formation of fasciated fruits. Nevertheless this phenotype was no more observed in following analysis. We hypothesize that the *Sl-IAA29* regulation is strikingly linked to environmental conditions which remain to be determined. Moreover, we have identified the presence of an upstream ORF (open reading frame, uORF) of nine amino acids in the 5'UTR sequence of *Sl-IAA29*. Previous reports have all shown that uORF are cis-regulators of gene expression by notably blocking ribosomal activity. Interestingly, preliminary analysis showed that the uORF in the *Sl-IAA29* 5'UTR can negatively trans-regulating Sl-IAA29. Nevertheless mechanism involved in this regulation process remains to be elucidated.

To get insight on Aux/IAA function notably regarding flower and fruit development it would be interesting to characterize other genes. Nevertheless *Sl-IAA* over-expression plants displayed no phenotypes regarding fruit development. Due to long time necessary to generate transgenic plants it would be interesting to identify by TILLING *Aux/IAA* mutants. Because of its specific behavior notably due to the absence of conserved II the investigation of physiological significance of *Sl-IAA32* would be of particular interest.

To date, the dynamic analyse of auxin distribution and signalling during tomato fruit development are poorly investigated. Recently, a novel Aux/IAA-based auxin signalling sensor termed DII-VENUS was engineered in the model plant *Arabidopsis thaliana* (Brunoud et al., 2012). This new tool provides a map of relative auxin distribution at cellular resolution. Some experiments using DII-VENUS in tomato are actually in progress in the lab. The high-resolution spatio-temporal information about auxin distribution and response during tomato fruit development would bring better understanding of auxin function in this process.

Despite that *Sl-IAA27* and *Sl-IAA9* seem regulate fruit development in distinct manner, the over-expression of *Sl-IAA9* in the *Sl-IAA27* down-regulated plants suggests that *Sl-IAA9* and *Sl-IAA27* could be at least in part functionally redundant. To be able to conclude on this, crossing between *Sl-IAA27* RNAi plants and *Sl-IAA9* down-regulated plants could be performed. In addition, structure analysis of Sl-IAA27 and Sl-IAA9 revealed a conserved motif YxGLS at the N-amino terminal part before the domain I. This domain was found in Sl-IAA27 and Sl-IAA9 homologs in all species studied. To determine if this domain has a functional significance several experiments could be performed. Chimeric forms of Sl-IAA27 and Sl-IAA9 proteins without this YxGLS domain can be constructed. Complementation of *Sl-IAA27* and *Sl-IAA9* down-regulating plants with chimeric protein forms respectively may indicate if we are able to restore wild-type plants phenotype and therefore if YxGLS domain has a functional significance regarding fruit development.

To understand the functional differentiation among the *Aux/IAA* family in tomato, the determination of qualitative and quantitative interactions between Aux/IAAs and their ARF partners will be required. Moreover elucidation of repression mechanism by which Aux/IAA domain I works would be a clue element to determine. Indeed, several lines of evidence in the

literature support a model for EAR motif-mediated repression (similar to domain I) acting via epigenetic mechanisms resulting from chromatin modifications (Kagale and Rozwadowski 2011). Moreover auxin interacts with other hormones to regulate fruit development such as cytokinins and gibberellins and the regulation of Aux/IAAs expression by these hormones should be investigated.

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